



Silja Mentula

# Analysis of Canine Small Intestinal and Fecal Microbiota

Prevention of Ampicillin-induced Changes with Oral  $\beta$ -lactamase

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National Public Health Institute  
Helsinki, Finland  
and  
Department of Applied Chemistry and Microbiology  
University of Helsinki, Finland

**Silja Mentula**

**ANALYSIS OF CANINE SMALL INTESTINAL AND FECAL  
MICROBIOTA**

**PREVENTION OF AMPICILLIN-INDUCED CHANGES  
WITH ORAL  $\beta$ -LACTAMASE**

**ACADEMIC DISSERTATION**

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National Public Health Institute, Helsinki, Finland

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To my family

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## ABBREVIATIONS

CFU	colony forming unit
DNA	deoxyribonucleic acid
GALT	gut-associated lymphoid tissue
IBD	inflammatory bowel disease
i.v.	intra venous
MAC	microflora-associated characteristics
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
NCCLS	National Committee for Clinical Laboratory Standards
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
p.o.	per oral
rRNA	ribosomal ribonucleic acid
SIBO	small intestinal bacterial overgrowth
TGGE	temperature-gradient gel electrophoresis
TEM	class of plasmid-mediated $\beta$ -lactamases (first isolated from a patient named Temoniera)
TRBL	targeted recombinant $\beta$ -lactamase
VRE	vancomycin-resistant enterococci



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## ABSTRACT

**Background:** The intestinal microbiota affects many physiological and immunological functions of the body and is closely connected to the homeostasis and health status of an individual. Due to difficulties in obtaining samples from the intestine and studying complex microbial populations the knowledge of the composition, dynamics and metabolic potential of microbial populations in the upper gut is limited. Antimicrobials may disrupt the balance of the microbiota by causing quantitative and qualitative changes such as emergence and enrichment of antibiotic-resistant populations, and decrease in colonisation resistance against pathogens. The aims of this study were to analyse cultivable jejunal microbiota of a dog in detail and compare it to faecal findings, and to evaluate the ability of oral  $\beta$ -lactamase administration, targeted to degrade intestinal ampicillin residues, to prevent ampicillin-induced changes in the intestinal microbiota.

**Material and methods:** Jejunal and faecal samples were obtained from healthy laboratory beagles with permanent jejunal fistula randomised into 3 treatment groups receiving ampicillin, ampicillin +  $\beta$ -lactamase or placebo. Samples were collected before, during and after the treatment and analysed using bacterial culture and TGGE (temperature-gradient gel electrophoresis) for total bacterial counts and composition of the dominant microbiota. Susceptibility testing and PCR for detection of TEM gene were the methods for monitoring resistance rates. In addition, jejunal and serum ampicillin concentrations were determined.

**Results:** The relative quantities of predominant bacterial groups, proportion of anaerobes, intra- and inter-individual species variety and stability of the microbiota differed significantly between the jejunum and faeces. Oral  $\beta$ -lactamase inhibited the effects of ampicillin, monitored as significant differences in resistance rates and

bacterial composition during treatment between the groups receiving ampicillin with and without  $\beta$ -lactamase.

**Conclusion:** This study suggests that faeces with stable and rich microbiota does not represent jejunal microbiota that is characterised as harbouring few individually varying bacterial species at a time with fluctuating counts. Oral  $\beta$ -lactamase has potential in inhibiting the adverse effects of  $\beta$ -lactam therapy.

**Keywords:** gastrointestinal tract, jejunum, microbiota,  $\beta$ -lactams, susceptibility

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## TIIVISTELMÄ

**Taustaa:** Suolistomikrobisto vaikuttaa moniin kehon fysiologisiin ja immunologisiin toimintoihin ja siten myös kehon tasapainotilaan sekä yksilön terveyteen. Johtuen vaikeuksista näytteiden saamisessa suoliston eri osista sekä monimuotoisten mikrobiopopulaatioiden tutkimisen hankaluuksista, ymmärtämys suolistomikrobiston koostumuksesta, dynamiikasta ja metaboliikasta on vaillinaista. Antibiootit voivat häiritä mikrobiston tasapainoa aiheuttaen niin määrällisiä kuin laadullisia muutoksia mikrobistossa kuten antibiootille vastustuskykyisten mikrobien ilmaantumista ja rikastumista ja taudinaiheuttajamikrobien kolonisaatioresistenssin alenemista. Tämän tutkimuksen tavoitteena oli analysoida koiran ohutsuolen mikrobisto yksityiskohtaisesti viljelymenetelmää käyttäen ja verrata sitä ulosteen mikrobistoon. Lisäksi tavoitteena oli arvioida suun kautta annettavan, suolistoon kulkeutuvien  $\beta$ -laktaamiryhmän antibioottijäämien hajoittamiseen suunnitellun  $\beta$ -laktamaasivalmisteen tehoa ampisilliinin suolistomikrobistossa aiheuttamien muutosten ehkäisyssä.

**Materiaalit ja menetelmät:** Ohutsuolen sisältö ja ulostenäytteet kerättiin terveiltä beagle-koirilta, joille oli tehty pysyvä fisteli (permanent jejunal fistula). Koirat oli satunnaistettu kolmeen hoitoryhmään, jotka saivat joko ampisilliiniä, ampisilliiniä ja  $\beta$ -laktamaasia tai lumevalmisteita. Näytteet kerättiin ennen antibioottikuuria, kuurin aikana ja sen jälkeen ja analysoitiin käyttäen viljelyä ja TGGE-profilointia (temperature-gradient gel electrophoresis) bakteerien kokonaismäärien ja koostumuksen tutkimiseen. Antibioottiresistenssin esiintymistä seurattiin herkkyysmääryksin sekä TEM-geenin (plasmidi-välitteinen  $\beta$ -laktamaasigeeni) esiintymistä PCR-määryksin. Lisäksi seurattiin ohutsuolen sisällön sekä seerumin ampisilliinipitoisuuksia.

**Tulokset:** Vallitsevien bakteeriryhmien suhteelliset osuudet, anaerobisten bakteerien osuus, yksilöiden sisäinen sekä yksilöiden välinen lajivaihtelu ja mikrobiston stabiilius erosivat merkittävästi ohutsuolen ja ulosteen mikrobistojen välillä.  $\beta$ -laktamaasivalmiste ehkäisi ampisilliinin vaikutuksia: teho havaittiin merkittävinä eroina resistenssin esiintymisessä ja mikrobiston koostumuksessa ampisilliinia ja ampisilliiniä sekä  $\beta$ -laktamaasivalmistetta saaneiden ryhmien välillä.

**Johtopäätökset:** Tämä tutkimus osoittaa, että ulosteen melko stabiili ja runsas mikrobisto ei edusta ohutsuolen rajoittunutta, yksilöittäin, ajallisesti, määrällisesti ja laadullisesti vaihtelevaa mikrobistoa.  $\beta$ -laktamaasi on lupaava valmiste  $\beta$ -laktamiantibioottien aiheuttamien epäsuotuisten vaikutusten ehkäisyyn.

Avainsanat: suolistomikrobisto, ohutsuoli,  $\beta$ -laktamit, mikrobilääkeherkkyys

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

- I**      Mentula S, Harmoinen J, Heikkilä M, Westermarck E, Rautio M, Huovinen P, Könönen E. Comparison between the cultured small intestinal and fecal microbiota in beagle dogs. *Appl Environ Microbiol* 2005, in press.
  
- II**     Harmoinen J, Mentula S, Heikkilä M, van der Rest M, Rajala-Schultz PJ, Donskey CJ, Frias R, Koski P, Wickstrand N, Jousimies-Somer H, Westermarck E, Lindevall K. Orally administered targeted recombinant Beta-lactamase prevents ampicillin-induced selective pressure on the gut microbiota: a novel approach to reducing antimicrobial resistance. *Antimicrob Agents Chemother* 2004;48:75-79.
  
- III**    Mentula S, Harmoinen J, Koski P, Westermarck E, Rautio M, Huovinen P, Könönen E. Inhibition of ampicillin-induced emergence of resistance in intestinal coliforms by targeted recombinant beta-lactamase. *Int J Antimicrob Agents* 2004;24:555-561.
  
- IV**    Mentula S, Virtanen T, Kanervo-Nordström A, Harmoinen J, Rautio M, Huovinen P, Könönen E. Relatedness of *Escherichia coli* strains with different susceptibility patterns isolated from beagle dogs during ampicillin medication. (submitted for publication)

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## **Author's contribution**

### **Publication I**

Silja Mentula (SM) was responsible for the microbiological experimental work and carried out identification of the strains, excluding 16S RNA gene sequencing. Collected and interpreted the results and wrote the paper.

### **Publication II**

SM was responsible for the culture-based experimental work, including the selection of coliform isolates and collecting and modifying the susceptibility data. Interpreted the culture-based results and wrote the paper together with the corresponding author.

### **Publication III**

Responsibilities in experimental work were similar to publication II. SM analysed the resistance patterns, interpreted the results and wrote the paper.

### **Publication IV**

SM was responsible for the microbiological experimental work, analysed and compared the typing data to susceptibility data, interpreted the results and wrote the paper.

## INTRODUCTION

The intestinal microbiota is known to have a crucial impact on the health status of an individual. Microbes influence functions such as nutrition, development and maintenance of the immune system, and defence against pathogens. To understand the relationship between the intestinal microbiota and health, and the metabolic potential of the microbes, and to evaluate the effect of different diets, treatments or medical conditions on the microbiota, fundamental knowledge of the intestinal microbial populations is needed.

Because of the inaccessibility of the intestine, there are major difficulties in studying the intestinal microbiota in its natural habitat and most studies of the intestinal microbiota have been done using faecal samples. Due to low number of studies data on the differences between the microbiota of different parts of the gut are limited. The microbiota of the human upper gut and caecum has been reported to differ significantly from that of the colon and faeces (Gorbach *et al.* 1967, Justesen *et al.* 1984, Simon & Gorbach 1986, Marteau *et al.* 2001). However, the recoverability of small intestinal microbiota from faeces has not been fully analysed.

Bacterial composition, activities and concentrations can be severely distorted by outside agents such as antimicrobials. Exposure of gut microbiota to antimicrobials selects resistant bacteria that may enrich and spread within a given surrounding (Livermore 2003, Münnich & Lübke-Becker 2004, Drazenovich *et al.* 2004). Increasing rates of antimicrobial resistance following extensive use of antimicrobials has led to a worldwide interest in finding ways to inhibit the emergence of resistance.

The present study was done in order to make a detailed analysis on the canine jejunal microbiota and compare it with corresponding faecal microbiota in 22 beagle dogs with permanent jejunal fistula. The other aim of the study was to analyse the effect of i.v. ampicillin treatment on the intestinal microbiota, especially on the antimicrobial resistance of coliforms, and to analyse whether the adverse effects of ampicillin could be inhibited by simultaneous oral administration of  $\beta$ -lactamase, designed to degrade antibiotic residues in the gut. The analyses were performed using culture method and

various molecular tools (TGGE, quantitative PCR, PFGE). The ultimate aim of the study was to contribute to the better understanding of the composition and dynamics of the gut microbiota, and to the development of means to prevent antimicrobial resistance.



## REVIEW OF THE LITERATURE

### 1. Gut microbiota in health and disease

#### 1.1. The importance of microbes

Microorganisms colonise all mucosal surfaces forming a complex and dynamic entities. The intestine harbours the vast majority of the microbes of the body, and there the microbes also have the most prominent impact on our health. The gut microbiota can be described to be in a state of unstable stability with reference to the relatively stable composition of the host specific main bacterial groups and their proportions within an individual, and to the simultaneous constant flow of new bacterial clones co-existing and replacing the previous ones in a balanced and seemingly homologous living system (McCartney *et al.* 1996, Franks *et al.* 1998, Zoetendal *et al.* 1998, Falk *et al.* 1998). Gut microbiota can be regarded as a metabolically active organ of the body, beneficial to both microbes and the host. Compared to skin, the contracting gastrointestinal tract with villi and microvilli comprises 100-fold surface area ( $2\text{ m}^2$  versus  $150\text{-}200\text{ m}^2$ , respectively) providing a considerable amount of space for digestive and physiological processes (Van Dijk 1997, Waldeck 1990). The vital importance of the microbiota to the homeostasis of the body is obvious since the imbalance of the microbiota may cause several different disturbances of the gastrointestinal functions such as diarrhoea and impairs the health status of an individual.

Microbiota has crucial role in many physiological/biochemical and immunological functions of the body (Gordon & Pesti 1971, Berg 1996). The importance of the microbiota has been analysed by comparisons of germ-free (sterile) and conventional animals, most often mice, rats, or chickens. Individuals grown in a microbe-free (sterile) environment lack many features of the immune system, require 30% more calories to maintain body mass and die sooner than those grown in normal conditions (Gordon & Pesti 1971, Wostmann *et al.* 1983, Tanaka & Ishikawa 2004, Bäckhed *et al.* 2004). The features being altered by bacterial activity, differing between germfree and conventional mice, are collectively known as microflora associated characteristics (MACs) (Midtvedt 1989). In turn living microbes in the intestine are provided a niche

with abundance of nutrients and fermentable carbon sources originating from host secretions, diet, cast off epithelial cells and mucin. Additionally, the conditions are stable, warm and humid.

## **1.2. The effects of intestinal microbiota**

To colonise different niches of the intestinal tract microbes have acquired distinct and appropriate adaptation strategies. The commensal and symbiotic (indigenous) microbes are those that are beneficial or at least not harmful to the host in contrast to the harmful pathogenic (transient) species. In addition to the immunopotentiality (maturation of the gut-associated lymphoid tissue, GALT) the commensal bacteria play a significant role in the nutrition by degrading otherwise non-digestible food compounds, producing vitamins and short-chain fatty acids, used as an energy source by the colonic mucosa, conversion of urobilin to urobilinogen, conversion of cholesterol to coprostanol, and in absorption of ions (Moore & Holdeman 1974, Mackowiak 1982, Simon & Gorbach 1986). The gut-associated lymphoid tissue (GALT) contains over 80% of all B-cells (antibody producing lymphocytes) of the body and more lymphoid cells than the spleen, peripheral lymph nodes and blood together (Brandtzaeg *et al.* 1989). The increasing frequency of atopic allergies has been linked to overly hygienic living conditions, referred to as the hygiene hypothesis, altering the colonisation patterns in infancy and leading to lack of tolerance to harmless food proteins and antigens (Wold 1998). Indigenous bacteria also stimulate vascularisation and development of intestinal villi, have an important role in the metabolism of endogenous and exogenous compounds (detoxification), and in prevention of colonisation and proliferation of pathogens and opportunistic microbes (Hooper *et al.* 2002, Stappenbeck *et al.* 2002). Term colonisation resistance was introduced by van der Waaij *et al.* (1971). It provides non-specific defence against infections and includes various bacterial mechanisms such as competitive exclusion (competition of living/adhesion space and nutrients) and production of substances harmful to pathogens (antimicrobial agents, bacteriocins) as well as anatomical and physiological features of the host (mucosa, secretions, gastrointestinal motility). Most effects of bacteria against other bacteria are strictly local since e.g. bacteriocins are degradable by host digestive enzymes (Guarner & Malagelada 2003).

### 1.3. Dysfunctions of the gut microbiota in humans

#### Clinical disturbances

Whether the causes for dysfunctions in the intestine are of physiological or exogenous origin, they are often associated with gastrointestinal tract infections with altered bacterial composition, bowel movements, and/or water and ion balance. The clinical terms for gastrointestinal tract infections are gastroenteritis, diarrhoea, dysentery and enterocolitis, each with distinctive set of clinical manifestations (Mims *et al.* 1998).

One of the most common type of chronic gut dysfunction, the inflammatory bowel disease (IBD), has been linked to loss of tolerance to commensal bacteria. Intestinal bacteria may be involved in maintaining the inflammation reaction in IBD (e.g. ulcerative colitis, Crohn's disease) since patients have increased mucosal secretion of IgG antibodies against several commensal bacteria and have higher amounts of bacteria attached to their epithelial surfaces compared with healthy people (Macpherson *et al.* 1996, Swidsinski *et al.* 2002).

Another serious clinical condition possibly linked to disturbances in gut microbiota is colon cancer. By production of potentially toxic or carcinogenic substances such as amines, phenols and indols, intestinal bacteria are most likely one of the factors modulating the risk of initiation of colon cancer (Smith & Macfarlane 1996, Macfarlane & Macfarlane 1997). Increase in the incidence and growth rate of colonic tumors have been shown to be associated to some *Bacteroides* and *Clostridium* species while probiotic bifidobacteria bacteria prevent tumorigenesis in rats and mice (Onoue *et al.* 1997, Horie *et al.* 1999, Singh *et al.* 1997). Similarly, the presence of *Bacteroides vulgatus* and *Bacteroides stercoris* has been connected with a high risk of colon cancer whereas *Lactobacillus acidophilus*, *Lactobacillus* S06 and *Eubacterium aerofaciens* (currently *Collinsella*) associate with a low risk in humans (Moore & Moore 1995). Short-chain fatty acids produced by bacterial fermentation have been associated with reduction in the incidence of colon cancer and IBD (Wachtershauser & Stein 2000, Blottiere *et al.* 2003, Miyauchi *et al.* 2004).

### **Small intestinal bacterial overgrowth**

In small intestinal bacterial overgrowth (SIBO), also called the contaminated small bowel syndrome, total bacterial counts exceed those of healthy subjects by 1-2 log difference, usually crossing the limit of  $10^5$  cfu/g (Finegold & George 1989, Stotzer *et al.* 1998, Corazza *et al.* 1990). Patients with SIBO may have no symptoms and no predisposing factors, or may suffer from clinical manifestations such as malabsorption of lipids, amino acids and carbohydrates, vitamin B<sub>12</sub> deficiency, and disturbance in water and electrolyte transport in enterocytes (Gracey 1983). The causes and pathogenesis of the SIBO are poorly understood but several factors promoting manifestations have been identified. They include pathological abnormalities of the intestinal tract, stagnation of bowel contents, decreased gastric acidity, antimicrobial therapy and impaired immune system (Finegold & George 1989, Gracey 1983).

SIBO, as well as increased permeability of the intestinal mucosal barrier and deficiencies in host immune defences (e.g. mucin and secretory IgA production) are thought to promote bacterial translocation, i.e. the crossing of bacteria through the host epithelial mucosa (Berg 1999, Macpherson *et al.* 2000). Bacterial translocation enables bacteria to transfer within the body causing local infections, sepsis, shock, multisystem organ failure, or death of the host (Guarnier & Malagelada 2003). Delayed small intestinal transit times, possibly promoting small intestinal overgrowth, has been recorded in several conditions with high incidences of bacterial translocation such as obstructive pancreatitis, hepatic failure, and portal vein obstruction (Moody *et al.* 1995, Wang *et al.* 1994, Yi *et al.* 1999).

## **2. Composition of the human gut microbiota**

### **2.1. General features**

The mammal gut microbiota is composed of up to 1000 bacterial species with 30-40 species comprising the majority (99%) at a time, and with a particular individual combination of predominant species and unique strains distinct from other individuals (Savage *et al.* 1968, Savage 1977, Lee *et al.* 1971, Moore & Holdeman 1974, Xu & Gordon 2003).

The stability of the microbiota is challenged by gastric, pancreatic, and biliary acid secretions, peristaltic activity and continuous turnover of the epithelium and the mucus layer. On the other hand, peristaltic movement of the gut induces the exposure of different segments of the gut to the varied components of the intestinal contents ensuring the continuous beneficial interaction of microbiota, dietary molecules, epithelium and gut-associated immune system (Falk *et al.* 1998). The most numerous bacteria in the gut are obligatory anaerobic species accompanied by smaller amounts of aerobic/facultative bacteria with aerobes comprising only 1:100 - 1:1000 of the total count in the intestinal lumen (Finegold *et al.* 1983, Berg 1996). However, the proportion of aerobic species varies between the different regions of the intestinal tract.

**Table 1.** Microbial densities in different parts of the digestive tract (Simon & Gorbach 1984, Berg 1999, Marteau *et al.* 2001).

Site	Bacterial count (cfu/ml, cfu/g)
Oropharynx	$10^{8-11}$
Stomach	$0-10^4$
Small intestine	
Duodenum	$0-10^4$
Jejunum	$0-10^5$
Ileum	$10^{4-8}$
Caecum	$10^{6-8}$
Colon	$10^{10-12}$

Microbial densities are low in the stomach, and in the proximal and middle small intestine (duodenum, jejunum) because of the low pH and peristalsis (Table 1). The counts and diversity of the microbiota increase towards the distal small intestine (ileum) and colon (Savage 1977, Berg 1996). The small intestinal transit time is only 2-4 h compared with up to 60 h in the colon (Holzapfel *et al.* 1998, Drasar & Mill 1974). In the colon pH is higher than in the upper small intestine, the turnover is slower, redox potential is lower, short-chain fatty acid concentration is higher and the bacterial load is higher, indicating unique conditions in each gut compartment (Mackie *et al.* 1999). There is a shift in the biochemical atmosphere also within the colon between proximal colon with intense fermentation, rapid bacterial growth and pH of 5-6 and distal colon with lower fermentable substrate availability, putrefactive

processes, slow bacterial growth and neutral pH (Cummings & Macfarlane 1991, Tannock 1999a, Guarner & Malagelada 2003).

The conditions in the lumen differ also from those proximal to the mucosa. The gut can be divided crosswise into four different microhabitats: lumen, mucus layer, deep mucus layer and mucosal epithelial cells (Berg 1996). These microhabitats provide separate niches with unique properties. The predominant bacterial species associated to the jejunal, ileal and colonic mucosa differ significantly from the respective jejunal or ileal lumen and faecal bacterial community, and additionally bacterial counts between separate mucosal sites vary considerably (Bhat *et al.* 1980, Zoetendal *et al.* 2002, Wang *et al.* 2003). The mucosa-associated bacteria in colon and rectum are fairly similar in total counts and species composition (Poxton *et al.* 1997). The analysis of the mucosal microbiota is complicated by the undefined effects on the bacteria caused by the evacuation of the intestine preceding the biopsy collection and the possible washing procedures at sampling. A very limited number of studies has focused on the differences between the bacterial composition and activities in the different gut compartments.

## **2.2. Species composition**

The composition of the microbiota is known to be affected by several factors concerning the host such as age, diet, medication, health status, microbes in the environment and stress, as well as factors concerning the microbes such as growth rate, nutritional and adherence characteristics and microbial interactions (Holzapfel *et al.* 1998, Mitsuoka 1992). In addition, also host genetics have been reported to have significant correlations between bacterial communities (van der Merwe *et al.* 1993, Toivanen *et al.* 2001, Zoetendal *et al.* 2001). Once established, the microbiota remains relatively constant over time in adults (Finegold *et al.* 1983). Community shifts within the microbiota occur throughout the lifespan, especially in infants and in elderly people (Mitsuoka 1992, Hopkins *et al.* 2001, Favier *et al.* 2002).

As the composition of the microbiota is influenced by external factors such as acquisition of microbes in the diet, manipulating the microbiota from outside in order to improve the health of the host has drawn much attention (Mackie *et al.* 1999). The

concept of bacterial therapy using microbial cocktails has been introduced and it has shown to be a promising method to treat and prevent various respiratory and gastrointestinal tract infections (Huovinen 2001).

The cultivable dominating fecal bacteria belong to genera *Bacteroides*, *Bifidobacterium*, *Eubacterium* and related genera, *Clostridium*, *Peptococcus*, *Peptostreptococcus* and related genera, *Fusobacterium* and *Ruminococcus*, whereas the subdominant family and genera comprise of facultative anaerobes (aerobes) such as *Enterobacteriaceae*, *Streptococcus*, *Enterococcus*, *Lactobacillus* and *Proteus* (Table 2) (Simon & Gorbach 1984, Tannock 1999c). Some of these genera, especially *Eubacterium*, anaerobic gram-positive cocci and *Clostridium* have gone through extensive taxonomic revisions on the basis of new molecular methods (DNA-DNA hybridisation, 16S rRNA sequencing) that are replacing phenotypic methods as means to classify microbes. Molecular methods have also provided new aspects to the quantitative relations between different bacteria by determining proportions of group specific rRNA of total rRNA of a microbiota (Table 3). Attempts to combine culture-based data with molecular methods-based data has not yet provided us an accurate picture of the numerically predominant active species in the gut. Based on the partial sequencing of 16S rRNA, most of the bacteria found in the human colon fall into four phylogenetic clusters consisting of (1) *Bacteroides*, (2) *Bifidobacterium* and other gram-positive bacteria with high G+C content, (3) *Clostridium coccoides* and relatives (*Coprococcus*, *Eubacterium*, *Lachnospira*, *Ruminococcus*), and (4) *Clostridium leptum* and relatives, fusobacteria, and the *Atopobium* group (Wilson *et al.* 1997).

**Table 2.** Examples of common cultivable inhabitants of the adult human intestine (Moore & Holdeman 1974, Holdeman *et al.* 1976, Finegold *et al.* 1983, Sneath *et al.* 1986, Murray *et al.* 2003).

Genus / Group	Common representatives
<b>Anaerobic</b>	
<i>Bacteroides</i>	<i>B. fragilis</i> group, <i>B. splanchnicus</i>
<i>Clostridium</i> <sup>a</sup>	<i>C. perfringens</i> , <i>C. sordellii</i> , <i>C. bifermentans</i> , <i>C. innocuum</i>
<i>Bifidobacterium</i>	<i>B. adolescentis</i> , <i>B. longum</i> , <i>B. infantis</i> , <i>B. breve</i>
<i>Eubacterium</i> <sup>b</sup>	<i>E. rectale</i> , <i>Eggerthella lenta</i> , <i>Collinsella aerofaciens</i> , <i>Mogibacterium timidum</i>
Gram-positive cocci <sup>c</sup>	<i>P. anaerobius</i> , <i>P. micros</i> , <i>P. asaccharolyticus</i> , <i>Ruminococcus</i>
Gram-negative cocci	<i>Veillonella parvula</i> , <i>Acidaminococcus fermentans</i> , <i>Megasphaera elsdenii</i>
<i>Fusobacterium</i>	<i>F. necrophorum</i> , <i>Faecalibacterium prausnitzii</i> <sup>d</sup>
<b>Facultative/ aerobic</b>	
<i>Lactobacillus</i>	<i>L. acidophilus</i> , <i>L. casei</i> , <i>L. rhamnosus</i>
<i>Enterobacteriaceae</i>	<i>Escherichia coli</i> , <i>Klebsiella</i> , <i>Enterobacter</i>
<i>Streptococcus</i>	<i>S. bovis</i> , <i>S. anginosus</i> , <i>S. salivarius</i>
<i>Enterococcus</i>	<i>E. faecalis</i> , <i>E. faecium</i>
<i>Corynebacteria</i>	<i>Corynebacterium</i> , <i>Arcanobacterium</i> , <i>Actinomyces</i> , <i>Rothia</i>
Yeasts	<i>Candida albicans</i> , <i>Saccharomyces</i> , <i>Geotrichum</i>

<sup>a</sup> Divided into phylogenetic clusters (*C. coccoides*, *C. leptum*)

<sup>b</sup> Genera *Atopobium*, *Collinsella*, *Eggerthella*, *Mogibacterium*, *Slackia* have been reclassified from *Eubacterium*.

<sup>c</sup> Anaerobic gram-positive cocci includes genera *Anaerococcus*, *Coprococcus*, *Finegoldia*, *Micromonas*, *Peptococcus*, *Peptoniphilus*, *Peptostreptococcus*, *Ruminococcus*.

<sup>d</sup> Formerly *Fusobacterium prausnitzii*



**Table 3.** Mean proportions of different bacterial groups of the total microbial population in human feces detected by 16S rRNA hybridisation with group specific probes.

<b>Bacterial group</b> (probes)	Matsuki <i>et al.</i> 2002	Sghir <i>et al.</i> 2000	Rigottier-Gois <i>et al.</i> 2003	Franks <i>et al.</i> 1998
<b><i>Bacteroides</i></b> (Bacto1080, Bfa602, Bdis656, Bact338)	39%	37%	42%	20%
<b><i>Clostridium coccoides</i></b> (Erec482)	22%	14%	22%	29%
<b><i>Clostridium leptum</i></b> (Clept1240)	8%	16%		
<b>Low GC</b> (Strec493, Lacto722)		1%		12%
<b><i>Bifidobacterium</i></b> (Bif228, Bif164, Bif1412)	7%	<1%	3%	3%
<b>Enterobacteria</b> (Enter 1432)		<1%	1%	
<b><i>Prevotella</i></b> (Prevo0803)	6%			
<b><i>Faecalibacterium prausnitzii</i></b> (Fprau645)			9%	

### 2.3. Upper gut microbiota

In contrast to the heavily and steadily colonised lower regions of the intestinal tract, stomach and upper small intestine (duodenum, jejunum) harbour microbes in more time dependent manner. Both are practically free of cultivable living microorganisms when they are empty but harbour small numbers ( $10^{1-3}$  cfu/ml) of organisms at least transiently with highest numbers shortly after eating. The sparse microbiota in the upper small intestine is thought to consist mostly of oropharyngeal origin with major components being gram-positive acid tolerant lactobacilli, streptococci, fungi and staphylococci, and a minority of gram-variable veillonella, fusiforms, clostridia, actinomycetes, corynebacteria and haemophilus (Gorbach *et al.* 1967, Justesen *et al.* 1984, Berg 1996, Sullivan *et al.* 2003). The number of bacteria attached to the epithelia is also lower and consists of more gram-positive organisms compared to large intestine (Plaut *et al.* 1967, Guarner & Malagelada 2003). Whether the bacteria

found are only transient passengers or represent true colonisers of the upper small intestine is unclear.

In the distal small intestine (terminal ileum) the counts ( $10^{4-8}$  cfu/ml) and diversity, including appearance of substantial numbers of coliforms and bacteroides, increase drastically (Drasar & Mill 1975, Hentges 1993). The predominantly gram-positive bacteria of the upper small intestine shifts to gram-negative predominance towards the colon (Gorbach *et al.* 1967). In addition to declining acidity, the microbiota in the ileum is affected also by reflux of caecal contents (Gorbach *et al.* 1967, Nord & Kager 1984). The small intestine has been thought to harbour a distinct and characteristic microbiota but the composition of it has not been fully described. Ileal samples obtained by intubations and small intestinal samples obtained by needle aspiration grew total counts of  $10^{3-6}$  cfu/ml, with *Bacteroides* and anaerobic gram-positive rods such as *Bifidobacterium* dominating over enterococci and coliforms (Gorbach *et al.* 1967, Finegold *et al.* 1983). In the caecum aerobic species such as *Escherichia coli*, enterococci, and lactobacilli, comprise a significantly larger part of the total bacterial count ( $10^8$  cfu/ml), covering 50% of total bacterial rRNA, compared to faeces where aerobes cover 7% of the total count ( $10^{10}$  cfu/ml), respectively (Marteau *et al.* 2001).

## 2.4. Opportunistic commensals

Opportunistic commensals are the bacteria that are frequently present as permanent members of the microbiota but under favourable circumstances may proliferate vigorously and turn pathogenic. Although the vast majority of the normal microbiota consists of anaerobic bacteria most endogenous infections are caused by aerobes such as enterobacteria and enterococci (Vollaard & Clasener 1994). Common anaerobic genera such as *Bacteroides* and *Clostridium* include species with known pathogenicity; *B. fragilis* is the most frequent clinical isolate (Namavar *et al.* 1989) and *C. difficile* can exist as a harmless commensal in infants but cause disease especially in hospitalised elderly (Stark *et al.* 1982, Poxton 2005). Opportunistic bacteria may produce toxic metabolites causing disorders when accumulated. Some bacteria and fungi, e.g. species of *Neisseria*, *Bacteroides*, *Streptococcus* and *Candida* have the ability to change surface antigenicity in order to evade host immune responses (Deitsch *et al.* 1997, Kuwahara *et al.* 2004). Bacteria that are seldom

associated with infections such as *Bifidobacterium*, *Eubacterium* and *Lactobacillus* species can also occasionally cause serious illnesses (Brook & Frazier 1993).

### **Coliforms**

One of the predominating bacterial groups in the human and animal intestine is coliforms, i.e. gram-negative aerobic fermentative rods, with *E. coli* being the most common and most studied. It is also considered an indicator of faecal contamination in food and water. Although *E. coli* may not cover more than a fraction (<1%) of the total count of the intestinal bacteria, it is significant as a pathogen being one of the main causes of nosocomial infections, main causative of urinary tract infections and an important cause of diarrhoea (Eisenstein & Zaleznik 2000, Whittam *et al.* 1993, Maslow *et al.* 1995). *E. coli* has acquired several pathogenic mechanisms by which diarrhoeagenic strains can be divided into four distinct groups: enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC), and enteropathogenic (EPEC) *E. coli* (Mims *et al.* 1998). On the other hand, non-pathogenic *E. coli* may contribute to the preservation of colonisation resistance and has been given as bacterial probiotic therapy to patients with ulcerative colitis and in prevention of colonisation of antibiotic-resistant *E. coli* strains in premature babies and in prevention of neonatal calf diarrhea (Rastegar *et al.* 1990, Rembacken *et al.* 1999, Kruis *et al.* 2004, von Buenau 2005).

### **Fungi**

Fungi, most often yeasts, are normally present in small amounts in the intestine and faeces of mammals. In immunodeficient patients or when bacteria are suppressed by antimicrobials, yeasts (*Candida*, *Cryptococcus* spp.) may overgrow the intestine and act as pathogens causing diarrhoea, abdominal cramps and infections in other body sites (Krause *et al.* 2001, Tortorano *et al.* 2004). Yeasts can also be probiotic. In treating antibiotic associated diarrhoea *Saccharomyces boulardii* serine protease has been demonstrated to inhibit the pathogenic effects of *C. difficile* toxins A and B on human colonic mucosa (Castagliuolo *et al.* 1999).

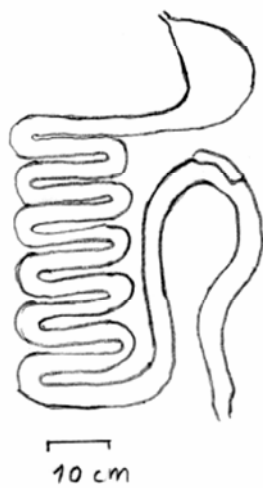
### 3. The digestive tract and intestinal microbiota of dog

The development of microbiota begins at birth when the sterile foetus is colonised in the birth canal and by the immediate environment. The bacterial succession is similar in the human and canine intestine with the very first colonisers originating from the mother, followed by microbes benefiting from breast-feeding and then drastically changing towards obligate anaerobes and greater diversity as solid foods are introduced (Benno *et al.* 1992a, Mackie *et al.* 1999). The most numerous colonisers in beagles during the first year are bacteroides, eubacteria, bifidobacteria, lactobacilli and anaerobic cocci while clostridia and streptococci increase later (Benno *et al.* 1992a).

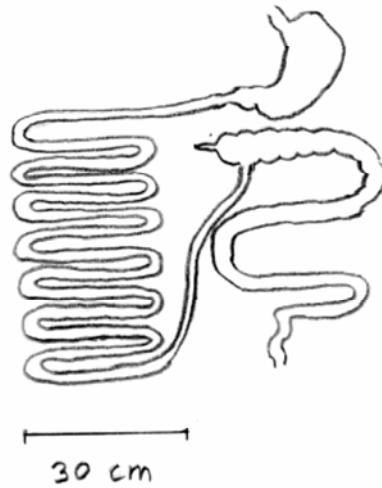
Since dogs are carnivorous the total length of their intestine in relation to the body length is somewhat shorter and the motility slower than in humans (Figure 1). As a whole the digestive tract of dog, however, resembles that of humans and the physiology is similar in many ways. Like humans dogs utilise intestinal microbiota in their physiology and both are homothermic mammals. The pH values in the different compartments of the digestive tract are also comparable to those of human: the pH in the dog stomach is 3, in duodenum and jejunum 6, in ileum 7.5, in colon 6.5 and in feces 6.2 (Smith 1965).

The main cultivable bacterial groups and most common findings in humans and dogs are the same including clostridia, bacteroides, streptococci, coliforms, enterococci, lactobacilli and veillonellae with increasing counts towards the large intestine (Smith 1965, Davis *et al.* 1977, Benno *et al.* 1992b, Greetham *et al.* 2002, Buddington 2003, Rinkinen *et al.* 2004). Most intestinal pathogens cause similar clinical symptoms in dogs as in humans (*C. difficile*, multiresistant gram-negatives, enterococci, staphylococci). Due to the ability of microbes to adapt and specify to niches with unique characteristics there are differences between the bacterial species level composition in different mammals. The most common intestinal dysfunction in dogs is diarrhoea. Another common disturbance, small intestinal bacterial overgrowth, composing of *E. coli*, enterococci and clostridia is associated with raised serum folate, reduced serum vitamin B12 concentrations and altered mucosal permeability and function (Batt *et al.* 1983, Rutgers *et al.* 1996).

**Figure 1.** Gastrointestinal tract of dog and human including stomach, small intestine, caecum and large intestine (Adopted from Stevens & Hume 1998).



**Dog**  
(*Canis familiaris*)  
Body length 90 cm



**Human**  
(*Homo sapiens*)  
Body length 180 cm

## **4. Obtaining samples from the intestine**

### **4.1. Getting representative samples from the upper gut**

Studies of microbiota *in vivo* carry some limiting factors associated to the complexity of the ecosystem leading to difficulties in identifying and determining the functions of all members or subpopulations. These include the unknown dynamic interactions within the microbiota and between microbes and host, inherent differences in the microbiota between study subjects, natural variability of the microbial strain composition with time, bacterial contamination during intervention trials between controls or staff and study group, and differences in human and experiment animal microbiota/physiology (Corpet 1987, Zoetendal *et al.* 2004).

Apart from general difficulties in analysing complex microbial populations, the difficulty in studying the gut microbiota lies in the inaccessibility of the microbiota in its natural habitat. Samples from intestinal contents from living healthy subjects obtained without anaesthesia and fasting, preoperative antibiotic prophylaxis, or laxatives/purgatives are simply not available. After any of these procedures the microbiota is no more in its natural state and may have gone through vast modification, and the sample size may also remain minimal. In addition, anaesthesia slows down the peristalsis, which makes backflow possible, and subjects studied at surgery have gastrointestinal dysfunctions and do not represent healthy individuals (Borriello *et al.* 1978). Attempts to get samples from the intestine without surgery include self-closing enterocapsules, tubes, endoscopes and, in animals, cannulation (Hirtzmann & Reuter 1963, Shiner *et al.* 1963, Gracey 1977, Hill 1996, Stotzer 1998). Capsules that pass through the intestine or tube that remains in place allow bacteria to multiply before the sample is processed. Invasive techniques going through other segments of the digestive tract carry the risk of contamination on the way. Tubes may also alter the intestinal motility and the position of the tube may be difficult to determine (Donaldson 1964).

Thus most analyses of the intestinal contents or biopsies are from the caecum or colon and originate from diseased persons (Gillian *et al.* 1992), sudden death victims (Macfarlane 1992) or are focused on certain probiotics (Alander *et al.* 1997, Alander

*et al.* 1999, Johansson *et al.* 1993) or sulphate reducers (Zinkevich *et al.* 2000). Very few studies have analysed the small intestinal microbiota of healthy subjects, and limited data are thus available (Marteau *et al.* 2001).

Because of these difficulties in obtaining proper samples from different parts of the intestinal tract, especially from the upper gut, and ease in collecting faecal samples, faeces is often the chosen sample type in studies of the gut microbiota. However, the faecal microbiota represents merely the distal colon, not even the proximal colon or terminal ileum, and may not represent the upper intestinal microbiota or the metabolic activities of it. Moreover, faecal predominant species tend to overgrow subdominant bacteria and the selectivity of selective agar media may be poor which makes it very difficult to gain isolates for further identification and to characterise the true bacterial diversity in faecal samples. Molecular methods with higher sensitivity can be used to detect the subdominant microbes but for further characterisation bacterial isolates should be obtained by culture.

#### **4.2. Jejunal fistula**

To overcome the inaccessibility of the intestine, a dog fistula model was designed to gain an easy access to jejunal chyme without causing pain to the animal or disturbing the normal functions of the gut (Wilsson-Rahmberg & Johnsson 1997, Harmoinen *et al.* 2001). The fistula is created using a 25 cm segment of the dog's own intestine by intussusception. The free ends of the intestine are connected by end-to-end anastomosis, and the isolated intestinal segment is inserted by end-to-side anastomosis to the intestine extending through abdominal cavity to connect the intestine to the skin with proximal end of the segment facing the skin. Small intestinal content (jejunal chyme) can be collected pre- or post-feeding without aspiration by inserting a silicon tube through the nipple valve into the intestine. A nipple valve can be surgically inserted to desired site at the small intestine. The closer the fistula is to the pylorus the more running (liquid) the content is (Harmoinen 2004). Most productive samples are obtained shortly after feeding (1-4 h) depending on the site of the fistula. Small intestinal samples from permanent fistula operated dogs can be obtained without any sedation/anaesthesia while the dog is fully active and standing. The fistula operation is reported not to have any significant effect on the intestinal motility, measured as

transit percentage of barium-impregnated polyethylene spheres, or on the cultivable microbiota (Harmoinen *et al.* 2001).

## **5. Analysing the microbiota**

### **5.1. Microscopic methods**

Microscopic methods can be valuable in analysing bacterial numbers (Kepner & Pratt 1994). However, microscopic counts do not necessarily correlate with the plate count. Plate count detects only reproducible cells whereas with microscopy it is possible to enumerate dead and/or viable cells depending on a stain applied (Breeuwer & Abee 2000). On the other hand, the clumping of cells and loss of cells after fixation during staining may distort microscopic counts. Often microscopy is combined with fluorescent in situ hybridisation (FISH), which enables the identification and enumeration of selected bacteria (Amann *et al.* 1995).

### **5.2. Culture method**

Traditionally culture method has been regarded as the golden standard for studying bacteria. It is notable that it is the only way to get bacterial isolates for further studies. The focus of the analysis of the intestinal microbiota has been on the enumeration and identification of numerically predominant cultivable faecal species (Holdeman *et al.* 1976, Finegold *et al.* 1983, Moore & Moore 1995). However, up to 60-80% of the mammal microbiota cannot be cultured and has not been characterised and identified (Moore & Holdeman 1974, Wilson & Blitchington 1996, Suau *et al.* 1999). The cultivable fraction is, however, relatively high compared to most other microbial ecosystems such as environmental biofilms and activated-sludge flocks (Amann *et al.* 1995). Uncultured microbes fail to grow on artificial agar media used in the culture as their suitable growth conditions, i.e. combination of nutrients, media, atmosphere, or necessary interactions with the host cells or other microorganisms, are not known or the cells are stressed or nonviable. In addition, the selective culture media used are either not selective enough or are too selective supporting the growth of only part of the microbes, and some microbes prevent the growth of others, and some are misidentified (Nelson & George 1995, Hartemink & Rombouts 1999). Thus the



culture result may not always give a truly representative picture of the microbiota studied. Culture method is also regarded as time-consuming and laborious. Despite the limitations, culture methods have successfully provided us the basic knowledge of clinically important microbes and intestinal ecosystem, and culture remains an indispensable tool for clinical microbiology.

The identification of isolated bacteria has traditionally relied on morphological, physiological and biochemical characteristics. This phenotypic characterisation based on cellular and colonial morphology, growth requirements and characteristics, fermentation profiles etc. has weaknesses connected to the poor reproducibility, ambiguity because of plasticity of bacterial growth and poor discriminatory power (McCartney 2002). Likewise, molecular-based genetic characterisation carries limitations. Thus there is often need to combine phenotypic with genotypic characterisation (polyphenic approach).

### **5.3. Molecular biological methods**

#### **Detection and identification**

Alternatively, culture-independent molecular methods have been introduced and notable amounts of DNA originating from uncultivable bacteria have been extracted, analysed and classified. Molecular methods are recognised as a standard phylogenetic classification tool (Stackebrandt *et al.* 1994). They may offer more rapid and reliable identification than culture because the identification is based on the composition of nucleic acids rather than on the genomic expression under given culture conditions, and the DNA can originate from living or dead cells (Nissen & Dainty 1995, Tannock *et al.* 2004). The applicability of molecular methods ranges from identification or detection of single bacterial species to characterisation of complex microbiotas, and molecular techniques have been applied in analysing evolutionary relatedness of different bacteria, in bacterial diversity studies, enumeration of bacterial groups, especially of extremely fastidious organisms, monitoring specific bacterial populations, and identification of bacterial isolates (McCartney 2002).

Molecular methods are based mainly on detection of ribosomal RNA (rRNA) or ribosomal DNA (rDNA; DNA encoding the rRNA). The probes for target DNA

sequences can be designed for detecting bacteria on different phylogenetic levels from major genera or the group level to the species or even strain-specific level depending on the type of the study ranging from gut ecology studies to tracking specific probiotics or pathogens (Charteris *et al.* 1997, Franks *et al.* 1998). Highly conserved regions of ribosome can be used for designing universal probes and different variable regions for specific/targeted probes. Several thousands of 16S rDNA sequences including numerous uncultured bacteria are freely available in genomic databanks. The target DNA can be detected using various PCR-based methods or dot blot hybridisation with specific synthetic oligonucleotide probes or using fixed bacterial cells by fluorescent in situ hybridisation (FISH) combined with flow cytometry or microscopic analysis (Amann *et al.* 1995, Wang *et al.* 1996, Wilson & Blichington 1996, Lin *et al.* 1997). Quantitative PCR allows quantification of all DNA fragments detected by PCR using specific controls of known quantity giving an estimate of the number of target microorganisms in the sample (Doungudomdacha *et al.* 2001, Sanz *et al.* 2004). Using multiplex PCR several target regions can be multiplied in single reaction with all necessary primers (García *et al.* 1998).

### **Population fingerprinting**

In general, fingerprinting techniques for studying bacterial communities within ecosystem are based on PCR and generate profiles representing the sequence diversity within the population (Zoetendal *et al.* 2004). Molecular fingerprinting techniques allow rapid assessment of the predominant bacterial species present in a sample and simultaneous analysis of multiple samples, and is well suited to studying changes in individual microbial communities over time (Suchodolski *et al.* 2004). Studies using rRNA are thought to reflect also the metabolic activity of bacteria; in hybridisation the ribosomal number is thought to be comparable to the number of active cells (Felske *et al.* 1997, Rigottier-Gois *et al.* 2003). TGGE (temperature gradient gel electrophoresis) or DGGE (denaturing gradient gel electrophoresis) can be used for creating bacterial profiles that can be compared using similarity percentages and dendrograms (Satokari *et al.* 2003, Muyzer *et al.* 1993). TGGE has high sensitivity for detecting sequence differences (Muyzer *et al.* 1993). The composition of individual rDNA genes can also be sequenced and analysed using cloning libraries (Suau *et al.* 1999) or profiled using probing grids, checkerboard hybridisation (O'Sullivan 1999) or DNA microarrays

(Wilson *et al.* 2002). Sequencing of single PCR clones is a laborious and expensive procedure but allows immediate discrimination between bacterial species.

Like culture, molecular methods carry limitations such as PCR biases, laborious processing at the species level, requirements for a clone library or sequence information, semi-quantitative results, and expensive machinery and reagents (Zoetendal *et al.* 2004). The biggest challenge in molecular methods lies in the quantification of different microbial groups and determining their functionality, activity and dynamics within the diverse microbial populations (Sghir *et al.* 2000, McCartney 2002).

#### **5.4. Molecular typing methods**

Molecular methods are widely used for typing of cultured isolates. The most common molecular typing methods are plasmid profiling, pulsed-field electrophoresis (PFGE), multilocus enzyme electrophoresis (MLEE), ribotyping and random amplification of polymorphic DNA (RAPD-PCR, AP-PCR) (O'Sullivan 2000, Tannock 2001). These typing methods are applicable to numerous bacterial genera and can be used to distinguish bacterial isolates and to determine their relatedness.

For epidemiologically related organisms PFGE has been proven a powerful discriminating tool, especially in food-born outbreaks and nosocomial infections (Grif *et al.* 1998, Barrett *et al.* 1994, Diekema *et al.* 1997). PFGE has high discriminatory power and can differentiate e.g. between subtypes of O157:H7 associated with specific outbreak investigations, though for large-scale screening of O157:H7 isolates other fingerprinting methods such as repetitive extragenic palindromes (REP) and Box primers (rep-PCR), amplified fragment length polymorphism (AFLP) and ribotyping techniques are also applicable and less time consuming (Hahm *et al.* 2003). In PFGE the bacterial DNA is digested with restriction enzyme(s), the obtained fragments are separated electrophoretically and discrete banding patterns for each isolate are analysed. Strains typed with PFGE using standardised methodology can be compared internationally using clone libraries. The determination of relatedness of non-related organisms with PFGE is more tricky and cannot use the same criteria as for related organisms, since the relationship between restriction fragment banding

pattern and true genetic relatedness is poorly understood. In addition, PFGE includes always subjective decisions such as the selection of restriction enzyme(s), reading the restriction fragment banding pattern and choosing methods for analysing the data, and the interpretation of the results (Davis *et al.* 2003, Singer *et al.* 2004). Distracting variabilities in the patterns brought by transferable large plasmids can be eliminated by excluding fragments smaller than 160 kb from the analysis (Guyot *et al.* 1999).

### **5.5. Indirect methods for analysing microbiota**

The microbiota, including cultivable and uncultivable species, can also be studied by indirect methods such as cellular fatty acid analysis (Peltonen *et al.* 1992), gas production (El Oufir *et al.* 1996), fermentation end product analysis (Topping & Clifton 2001) and enzyme activity profiling (Tannock 1999b, Gorbach & Bengt 1986). Analysis of microbial biochemical activities gives an insight to the functionality of the microbiota without having to go through culture, isolation and identification steps. The *in vitro* methods, such as continuous flow cultures or batch fermentations, may not always be predictive of the actual *in vivo* situation, since the complex and dynamic interactions between the gut and the microbiota are difficult to mimic. The analysis of microbiota using bacterial metabolites is also complicated by their unstability as they are used by other microbes or absorbed.

## **6. Antimicrobial agents**

### **6.1. Classification of antimicrobial agents**

Infectious diseases can be controlled by treating a disease with drugs (antimicrobial therapy), or by preventing it by vaccines (immunisation) and healthy environment (sanitation, hygiene, nutrition). The most important drugs for treating microbial diseases are antimicrobial agents, representing one of the great triumphs of modern medicine. Antimicrobial agents are chemical substances produced by microorganisms which are active against other microorganisms. In prokaryotes cell wall synthesis, protein synthesis (bacterial ribosome), nucleic acid synthesis and cell membrane function are sufficiently different from those in human eukaryotic cells so that they can be targets for antibacterial agents without inhibition of the equivalent mammalian cell target (Mims *et al.* 1998). Many antimicrobial agents are natural microbial metabolic products or derivatives of them, chemically modified to improve their properties. There are also totally synthetic antimicrobial agents. Some bacterial species are inherently resistant to some classes of antimicrobials because they lack a susceptible target, they are impermeable to the agent or they produce inactivating enzyme (Brock *et al.* 2003). Antimicrobial agents can be divided on the basis of the target site, chemical structure or function (Table 3).

### **6.2. Emergence of antimicrobial resistance**

Antimicrobial resistance is a worldwide problem and numerous strategies to avoid the emergence of resistance have been designed. New antimicrobials are being introduced but as most of them are similar in structure to the previous ones, the emergence of resistance is often only a matter of time. Despite of the development of a wide range of different types of antimicrobial agents, extremely adaptable bacteria seem to develop resistance to each new agent that comes along. Use of antimicrobials always causes selective pressure leading to reduction in efficacy, and the huge overuse induces bacteria to acquire resistance faster than expected (Livermore 2003). Inappropriate use of antimicrobials brings potential risk to the patient as risks of toxicity and opportunistic infection or superinfection, and to the community by selection of resistant organisms and increased health care costs. Both appropriate use

**Table 3.** Classification of antimicrobial agents on the basis of the target of action (Mims *et al.* 1998).

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**Inhibition of cell wall synthesis**

β-Lactams (ampicillin, cefotaxime, imipenem, aztreonam, cloxacillin)  
Glycopeptides (vancomycin, teikoplanin)

**Inhibitors of protein synthesis**

Aminoglycosides (gentamicin, streptomycin, kanamycin, neomycin, amikacin)  
Tetracyclines (tetracycline, doxycycline)  
Phenicol (chloramphenicol)  
Macrolides (erythromycin)  
Lincosamides (clindamycin, lincomycin)  
Streptogramins  
Fusidic acid

**Inhibitors of nucleic acid synthesis**

Sulfonamides  
Trimethoprim  
Quinolones (ciprofloxacin, norfloxacin, nalidixic acid)  
Rifamycin (rifampicin)

**Agents that affect DNA**

Nitroimidazoles (metronidazole)

**Inhibitors of cytoplasmic membrane function**

Polymyxins (colistin)

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of antimicrobials, including reduction in prescribing, and specific means to inhibit the emergence of resistance have been confronted. In addition to the mere use of antimicrobials the dissemination of resistance is connected to modern phenomena like large hospitals, institutional care of elderly, socialised care of children and youth in large units, and increasing travel (Holloway 2000, Sharma *et al.* 2005).

There is still a lot to learn about the dynamics in the emergence, spread, and maintenance of resistance. It remains unclear why some bacteria, called hypermutators, develop resistance very rapidly via mutation or DNA transfer while others remain susceptible, and why some resistant bacteria achieve epidemic spread while others do not. Potential factors in epidemic success include changes in bacterial properties such as adherence, desiccation and disinfectant tolerance, increased growth rate and adaptation to the fitness cost of resistance (Livermore 2003). When acquiring resistance by genomic alteration bacteria may lose some other character that may be

valuable when the antimicrobial selective pressure is absent. This burden or losing some feature is called fitness cost. Resistance mechanisms can be selected in both pathogenic and non-pathogenic bacteria, as shown in *E. coli* (Sunde & Sorum 1999). The largest reservoir of resistance has been thought to reside in the commensal gut microbiota (Guyot *et al.* 1999, Gulay *et al.* 2000, McDonald *et al.* 2001). Antimicrobial remains get into the intestine as residues that are not absorbed after oral administration, by diffusion from the surrounding tissue, and in bile via enterohepatic circulation (Nord & Heimdahl 1986b, Edlund & Nord 2000). After being enriched the resistance determinants may transfer within or between different bacterial genera e.g. between *Bacteroides* species and from gram-positive bacteria (*Clostridium*, *Streptococcus*, *Enterococcus*) to gram-negative bacteria (*Bacteroides*), and between bacteria originating from humans and animals (Nijsten *et al.* 1995, Shoemaker *et al.* 2001).

### **6.3. Mechanisms of resistance**

As there are several mechanisms by which antibiotics influence bacteria, there are also several mechanisms by which bacteria acquire resistance. Resistance may result from a single random chromosomal mutation or a series of mutations leading to synthesis of altered protein. Spontaneous mutants that have competitive advantage in the presence of antibiotic pressure survive and multiply. Dissemination of resistance is, however, more often the result of acquirement of transmissible plasmids, transposons or gene cassettes in integrons carrying resistance genes, typically coding for resistance to several unrelated classes of antimicrobials (Hall 1997, Poole 2001). Mechanisms of resistance include target site alterations (lowered affinity), alterations in the access to the target site (impermeability, efflux pumps, alternative metabolic pathways) and inactivation of the drug (enzymes that modify or destroy the antimicrobial) (Brock *et al.* 2003).

### **6.4. Adverse effects of antimicrobial agents**

An ideal antimicrobial agent would provide selective decontamination of the microbiota in a “gut friendly” manner, i.e. be active against pathogens and potentially pathogenic microbes but not against those that preserve the colonisation resistance.

Unfortunately only few, if any, available antimicrobials meet these criteria (Vollaard & Clasener 1994).

The use of antimicrobials leads to selection of resistant organisms among pre-existing variants and may radically reduce the number of commensal anaerobes susceptible to antimicrobials like *Bifidobacterium*, *Bacteroides* and *Lactobacillus* leading to reduction in colonisation resistance. This opens new niches for the remaining resistant opportunistic bacteria or transient pathogens that normally have restricted growth. They may overgrow the intestine or move to another anatomical site where the host is unable to tolerate the colonisation, and pathogenic microbes such as *C. difficile*, vancomycin-resistant enterococci, multidrug-resistant enterobacteria and *Candida* may proliferate (Berg 1996, Falk *et al.* 1998, Sullivan *et al.* 2001). In patients with *C. difficile*-associated diarrhoea treated with metronidazole the proportion of facultative bacteria was increased with higher enterobacterial and enterococcal counts but also with greater diversity of clostridia and lactobacilli compared to healthy subjects (Hopkins & Macfarlane 2002).

In addition to persisting infections and dissemination of resistant organisms, antibiotics predispose patients to serious clinical implications such as gastrointestinal disturbances and functional bowel symptoms, including diarrhoea, alterations in fermentation processes and reduction of short-chain fatty acid production (Bergogne-Berezin 2000, Sullivan *et al.* 2001, Maxwell *et al.* 2002). Antibiotic-associated reduced carbohydrate metabolism in turn may result in osmotic diarrhoea, poor absorption of fatty acids, water and electrolytes, altered bile acid balance and cholesterol metabolism (Hashimoto *et al.* 1996, Bergogne-Berezin 2000). Antimicrobials may also have allergic and toxic effects on the mucosa, and the pharmacologic effect that macrolides have on intestinal motility may affect the pathogenesis of antibiotic-associated diarrhoea (Bergogne-Berezin 2000). Antibiotics may also trigger release of LPS (lipopolysaccharides) endotoxins from gram-negative organisms with possible deleterious effects (Prins *et al.* 1985). A combination of oral *Candida albicans* administration and *Aspergillus fumigatus* spore exposure has been shown to induce allergic airway disease in mice treated with antibiotics (cefoperazone) but not in untreated mice (Noverr *et al.* 2004). In clinical practise, antimicrobial resistance increases mortality, morbidity and health care costs. If the



patient is not responding to the antimicrobial treatment, the length of hospital stay is prolonged or repeated physician visits are needed, and secondary antimicrobials are often more expensive than the first line choices (Livermore 2003).

### **Nosocomial infections**

Cross-contamination of resistant bacteria between individuals is especially significant in hospital environment where there is simultaneous selective pressure from several antibiotics and pathogens with lowered antimicrobial susceptibility may be enriched. In addition, a lot of patients with predisposing factors such as impaired immunosystem are exposed (Blazquez *et al.* 2000). Both hospitalised individuals and outpatients are reported to frequently carry resistant bacteria regardless of antibiotic usage (Levy *et al.* 1988). The most common organisms causing infections of the urinary tract, surgical wounds, skin, respiratory and gastrointestinal tract, as well as systemic infections are staphylococci including MRSA, enterococci including VRE, *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp., *C. difficile* and *Candida* spp. (Schaberg *et al.* 1991, Farr 2002, Biedenbach *et al.* 2004, Hull & Beck 2004, D'Agata 2004). However, in many cases the infective strain originates from the patient's own microbiota, thus hygienic measures cannot alone completely prevent nosocomial infections (Flynn *et al.* 1987), which are a widely acknowledged problem in both human and animal care, and are known to contribute significantly to mortality and health care costs. In Finnish patients over 50 000 nosocomial infections are reported annually, contributing to 2000-5000 deaths (Ora 2005).

### **6.5. Use of antimicrobials in humans and animals**

Common use of antimicrobials of human importance in cattle and pets may increase the risk of transferring resistance from animal to human microbiota. Controversial arguments about antibiotic use in animals are being debated (Piddock 1996, Cook 1997, Phillips *et al.* 2004, Guardabassi *et al.* 2004). Reductions in prescribing antimicrobials at a national level have been shown to reduce the prevalence of resistance within few years (Kristinsson 1997, Seppälä *et al.* 1997). However, reduction in antimicrobial usage does not always lead to reduced resistance. Resistant strains that are well adapted and carry minimal or no fitness cost because of the resistance determinant may not be displaced for a long period of time after the

exposure to antimicrobial agent has ceased. Strains that acquire resistance plasmid may initially grow more slowly than plasmid-free counterparts in the absence of selection pressure but after repeated subculture the difference may diminish as shown in tetracycline- and streptomycin-resistant *E. coli* and in tetracycline- and erythromycin-resistant *Bacteroides* (Bouma & Lenski 1988, Lenski 1997, Shoemaker *et al.* 2001).

## 6.6. $\beta$ -Lactam antibiotics

One of the most important groups of antimicrobial agents is  $\beta$ -lactam antibiotics, which are widely used in human and veterinary medicine (Livermore 1998).  $\beta$ -Lactams account for 60% of antimicrobial consumption in Finland (National Medical Statistics, [www.nam.fi](http://www.nam.fi)). Most commonly used  $\beta$ -lactams include penicillins (ampicillin, piperacillin, cloxacillin), cephalosporins (cephalexin, cefuroxime, ceftriaxone), cephamycins (cefoxitin) and carbapenems (imipenem, meropenem). Their antimicrobial potential is based on their ability to interfere in the biosynthesis of the peptidoglycan layer of the bacterial cell wall.

### Ampicillin

Ampicillin ( $C_{16}H_{19}N_3O_4S$ ) is relatively non-toxic and acid-stable and can be given orally or parenterally, parenterally in sodium salt form. Absorption of ampicillin is slower and more irregular than that of some of the esters (talampicillin) and analogs (amoxicillin) developed from it. The serum half-life of ampicillin in normal adults is 0.7-1.4h (Neuvonen *et al.* 1994). Peak serum concentrations are obtained at about 2h after oral administration and right after parenteral administration. After an oral dose 30% of ampicillin is excreted in urine within 6h, and after parenteral administration 75% (Kucers & Bennett 1987). Low amounts are excreted also in bile. Unexcreted ampicillin is inactivated mainly in the liver (Kucers & Bennett 1987).

## 6.7. $\beta$ -Lactamases

The most common mechanism of resistance to  $\beta$ -lactams is bacterial  $\beta$ -lactamases, produced by a number of both gram-negative and gram-positive bacterial species. In addition to clinically important enterobacteria,  $\beta$ -lactamase production has been described in various anaerobic genera of the normal microbiota such as *Bacteroides*, *Fusobacterium* and *Clostridium* (Nord 1986a).  $\beta$ -Lactamases inactivate the antibiotic by hydrolysing the amide bond in the  $\beta$ -lactam ring. Numerous different  $\beta$ -lactamases, both chromosomal and plasmid-mediated, have been described and they have been classified into four main groups and several subgroups on the basis of their function or structure (Livermore 1995, Bush *et al.* 1995, Bush & Jacoby 1997). Although hyperproduction of wild type  $\beta$ -lactamase induces resistance, the major contributors to resistance are thought to have arisen from single or multiple mutations in the structural  $\beta$ -lactamase genes (Reguera *et al.* 1991, Henquell *et al.* 1995, Belaouaj *et al.* 1994, Brun *et al.* 1994). Resistance determinants are transferable and are found within a wide variety of plasmids (Thomson *et al.* 1993). In hospital environment ampicillin is considered a significant factor in maintaining selection pressure for the most prevalent plasmid mediated  $\beta$ -lactamases in gram-negative organisms (Burman *et al.* 1992).

The predominant  $\beta$ -lactamases in gram-negative bacteria are TEM-type  $\beta$ -lactamases, coded by *bla*<sub>TEM</sub> genes, with TEM-1, TEM-2 and SHV-1 enzymes being the most common ones (Brinas *et al.* 2002). In *E. coli* TEM-1 is the predominating  $\beta$ -lactamase (Huovinen *et al.* 1988). These Ambler class A enzymes have traditionally been active against a broad spectrum of penicillins but evolution and selective pressure have conferred resistance to expanded-spectrum cephalosporins (extended-spectrum  $\beta$ -lactamases; ESBLs) or to  $\beta$ -lactam-clavulanic acid combinations (inhibitor-resistant TEM; IRT) or to both (complex mutant TEM; CMT) (Medeiros 1997, Sirot *et al.* 1997, Poirel *et al.* 2004).

## 6.8. Inhibition of $\beta$ -lactam resistance

### $\beta$ -Lactamase inhibitors

A way to avoid the microbial breakdown of  $\beta$ -lactams is the use of  $\beta$ -lactamase inhibitors simultaneously with  $\beta$ -lactams.  $\beta$ -Lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam contain  $\beta$ -lactam ring as substrates for the  $\beta$ -lactamase to which the “suicide inhibitor” binds preventing the enzyme to destroy the antibiotic. By themselves  $\beta$ -lactamase inhibitors have only little therapeutic value. Bacteria are, however, acquiring mechanisms to overcome the action of many of these inhibitors and a constant designing of new inhibitors is required.

### Degradation of intestinal $\beta$ -lactam residues

Another strategy to fight the emergence of resistance is intra-intestinal inactivation of antimicrobials by degrading the leftover antibiotic accumulating in the intestine where it triggers resistance and quantitative changes within the abundant microbial population (de Vries-Hoepers *et al.* 1993, Van der Waaij & Nord 2000). By degrading the antibiotic residue the selective pressure is abolished and the gut microbiota and colonisation resistance remain unaffected during antimicrobial treatment.

One way to bring  $\beta$ -lactamase to the intestine is by administrating  $\beta$ -lactamase-producing bacteria into the gut. Many intestinal microbes produce  $\beta$ -lactamase and faecal preparations from healthy subjects have been shown to inactivate  $\beta$ -lactams with individual variation (Veringa & van der Waaij 1984, Welling & Groen 1990). Patients harbouring  $\beta$ -lactamase-producing intestinal bacteria have less  $\beta$ -lactam-induced alterations in their microbiota (van der Waaij *et al.* 1986, Chachaty *et al.* 1992, Chachaty *et al.* 1993, Stark *et al.* 1995). In gnotobiotic mice detectable amounts of  $\beta$ -lactamase and inhibition of ceftriaxone activity were produced in the intestine by colonising the mice with a mixture of four  $\beta$ -lactamase-producing anaerobes (*Bacteroides thetaiotaomicron*, *Clostridium clostridioforme*, *B. uniformis* E9, *B. uniformis* V4E3) (Leonard *et al.* 1989).

Administrating the  $\beta$ -lactamase as a purified enzyme enables accurate screening and evaluation of the effects of the administration providing knowledge of the dosage.

When given orally the enzyme should be in a form where it does not affect the therapeutic effect of the target antimicrobial, thus it should be active only in the intestine, and not be absorbed into the systemic circulation, and, on the other hand, resist proteolysis and remain active in the intestine. This can be achieved in oral administration by packing the enzyme in capsules that are targeted to release the enzyme in the lower gastrointestinal tract and not in the stomach, in e.g. pH-based manner. In the present study, we analysed the ability to inhibit antibiotic-induced changes in the gut with a product with the above-mentioned characteristics, called targeted recombinant oral  $\beta$ -lactamase (TRBL). TRBL has been shown to degrade ampicillin in the jejunum of beagle dogs in a dose dependent manner but not to enter the systemic circulation or affect the serum ampicillin levels after i.v. administration of ampicillin (Harmoinen *et al.* 2003). A commercially available  $\beta$ -lactamase is in use in animal husbandry to destroy penicillin residues in cow milk (Korycka-Dahl *et al.* 1985, Suomen eläinlääkkeet 2003)

## **6.9. Testing antimicrobial susceptibility**

The antimicrobial susceptibility of bacteria can be determined *in vitro* by standardised methods such as disk diffusion method and broth or agar dilution assay (NCCLS 2002). Disk diffusion method is convenient in screening of resistance phenotypes (presence of the resistance genes) while dilution assay determines the precise level of resistance. In disk diffusion method the size of growth-inhibition zone around a filter paper disk containing the antibiotic on agar plate determines the susceptibility of the culture, and in antimicrobial dilution assay minimal inhibitory concentration (MIC) is determined as inhibition of growth within a series of antibiotic dilutions in broth or on agar. MIC can also be tested on agar using commercial E-test strips containing stable increasing antibiotic gradient producing a growth-inhibition ellipse.

## AIMS OF THE STUDY

The purpose of the study was to examine in detail the microbiota in the upper and lower part of canine intestine. The samples were collected before, during and after ampicillin treatment with or without simultaneous  $\beta$ -lactamase administration and analysed using both traditional culture method and molecular methods. When designing the study we hypothesised that (1) jejunal and faecal microbiota differ significantly from each other, (2) ampicillin administration has dramatic effects on the composition and counts of the microbiota and on the susceptibility of the coliforms, (3) oral targeted  $\beta$ -lactamase inactivates intestinal ampicillin, inhibits the ampicillin-induced changes and preserves colonisation resistance, (4) shifts in coliform population can be followed using resistance data and PFGE (pulsed-field gel electrophoresis) typing.

The specific aims of the study were to:

- (I) Compare the cultivable canine microbiota in the jejunum to that in faeces.
- (II) Analyse the ability of  $\beta$ -lactamase administration to prevent ampicillin-induced emergence of resistance in dogs during ampicillin treatment monitored by performing faecal bacterial culture combined to susceptibility testing, performing TGGE (temperature gradient gel electrophoresis) for dominant faecal bacterial groups and by determining the quantity of TEM-type  $\beta$ -lactamase-producing faecal bacteria by PCR and by following ampicillin levels in serum and jejunum.
- (III) Characterise canine jejunal and faecal coliform populations, their species variability and resistance patterns in the three treatment groups.
- (IV) Determine the genetic relatedness of coliform populations in the three treatment groups monitored by resistance patterns and PFGE typing.

## MATERIALS AND METHODS

### 1. Study design

In the present study, healthy male laboratory beagles with permanent jejunal fistula were used. The dogs were obtained from Harlan-Winkelmann GmbH (Borchen, Germany) and National Laboratory Animal Center (University of Kuopio, Finland). Their age was between 1 and 3 years and weight between 12 and 19 kg. The dogs were fistula operated and kept in the premises of the Department of Clinical Veterinary Sciences (Faculty of Veterinary Medicine, University of Helsinki). An intussuscepted nipple valve fistula was surgically inserted 170 cm distal to the pylorus (Harmoinen 2004). The dogs were kept individually, fed with commercial dog food (Pedigree®, Waltham®, Masterfoods, Helsinki, Finland) and taken for walks in the same premises. Coprophagy was avoided by cleaning faeces from the premise after each dog had been taken out. The local ethics committee for animal experiment action in Helsinki, Finland approved the study.

For a pilot study 6 beagles were randomised into 3 treatment groups, 2 in each, receiving ampicillin and placebo, ampicillin and targeted recombinant  $\beta$ -lactamase (TRBL) or only placebo. For the final study a new group of 18 beagles were similarly randomised into 3 treatment groups, 6 in each, but instead of 20 mg/kg of ampicillin for 10 days they received 40 mg/kg for 14 days (Table 1). The baseline samples were collected on day 0 and the follow-up samples 2 weeks after the treatment period (Table 2). Ampicillin sodium (A-PEN®, Orion Pharma, Espoo, Finland) or placebo (Natrosteril®, Orion Pharma) was administered i.v. 4 times a day 30 min after feeding and TRBL or placebo capsules were administered 20 and 27 min after feeding. Because of the different dosing of the ampicillin, the pilot study samples were excluded from the resistance studies (II, III, IV) but all baseline samples and all consecutive samples from the dogs receiving only placebo were included in the comparison study (I).

**Table 1.** Dosages used in the resistance studies for the 18 beagles.

Treatment	Amp / Pla	Amp / TRBL	Placebo
Ampicillin (i.v.)	40 mg/kg	40 mg/kg	0
TRBL (p.o.)	0	0.6 mg/kg	0
Number of dogs	6	6	6

**Table 2.** Sample collection timetable.

Time point	Day
Baseline	0
During treatment	4, 10, 14
Follow-up	28

### **TRBL (targeted recombinant $\beta$ -lactamase)**

In the designing of the oral  $\beta$ -lactamase the recombinant  $\beta$ -lactamase (Pen P protein) of *Bacillus licheniformis* 749/C was used as a model enzyme (Neugebauer 1981). The purified and freeze-dried targeted recombinant  $\beta$ -lactamase (TRBL) enzyme (Ipsat Therapies Ltd., Espoo, Finland) was released in gastrointestinal tract from enteric-coated pellets in a time and pH-controlled form (Röhm GmbH & Co KG, Pharma Polymers, Darmstadt, Germany) (Harmoinen *et al.* 2003). The capsule is targeted to dissolve above pH 5.5, aimed to release the enzyme in the jejunum.

## **2. Study animals and sample collection**

For study **I** jejunal and faecal baseline samples from 6 dogs in the pilot study and 16 dogs in the final study were included, 2 dogs from the final study with no jejunal samples were excluded. In addition, all samples from 7 of the dogs receiving placebo treatment were included. For study **II** all faecal samples from the 18 beagles recruited for the final study were included. For studies **III** and **IV** all jejunal and faecal samples from the 18 beagles recruited for the final study were included.

The serum and jejunal samples were collected 1 h after the first antibiotic administration of the day. The faecal samples were collected within 1 h of defecation. All samples were immediately frozen at -70°C. A total of 92 jejunal and 102 faecal samples were collected. The jejunal samples were not available from 2 dogs. The samples were processed in blinded manner, i.e. the Anaerobe Reference Laboratory personnel did not know from which treatment group the samples originated.



### 3. Study location

The dogs were operated and kept at the Department of Clinical Veterinary Sciences, Faculty of Veterinary Medicine, University of Helsinki, Finland. Parallel samples were analysed simultaneously using different methods in several different locations (Table 3). Bacterial culture, coliform identification, susceptibility testing and PFGE were performed at the Anaerobe Reference Laboratory, Department of Bacterial and Inflammatory Diseases, National Public Health Institute, Helsinki, Finland, whereas TGGE and PCR for TEM were performed at Microscreen BV, Groening, The Netherlands. Ampicillin concentrations in serum and jejunal samples were analysed at Yhtyneet Laboratoriot Oy (United Laboratories Ltd.), Helsinki, Finland. Statistical analyses were performed at each corresponding study location excluding Yhtyneet Laboratoriot.

**Table 3.** Methods used.

Method	Target	Sample material
Bacterial culture	Bacterial profiles, total counts, group/species level identification	Jejunal chyme, faeces
Susceptibility testing	Antibiograms	Jejunal and faecal coliform isolates
PFGE typing	Genetic relatedness	Jejunal and faecal <i>E. coli</i> isolates
TGGE	Bacterial profiles	Faeces
TEM-PCR	Amount of resistance gene	Faeces
High performance liquid chromatography (HPLC)	Ampicillin concentration	Jejunal chyme, serum

### 4. Bacterial culture

All samples from each animal were processed in parallel in order to ease the comparison between the 2 sample types and between consecutive samples. Small intestine fluid and faeces were thawed and homogenised, pH was measured (Benchtop 420 pH Meter, Orion) and the sample consistency, colour and odour were recorded. The homogenates were serially diluted ( $10^{-1}$ - $10^{-7}$ ) in prerduced peptone-yeast extract broth with pH 7.0. An extensive series of dilutions together with the undiluted sample (10 µl or 100 µl) were plated onto several non-selective and selective agar media and incubated at 35°C as appropriate (Table 4). Anaerobiosis was induced in anaerobic

**Table 4.** Culture media used. Presented ten-fold dilutions were used for faecal samples, for jejunal samples a limited set of dilutions at a lower dilution level was used.

Agar medium	Enrichment or selection	Bacteria	Dilutions	Atmosphere	Reference
Brucella	Sheep blood, hemin, vitamin K <sub>1</sub>	Total anaerobic counts	0, -4, -6, -8	Anaerobic	Jousimies-Somer <i>et al.</i> 2002
BBE	Bile, esculin, gentamycin	<i>Bacteroides</i> and <i>Bilophila</i> spp.	0, -4, -6	Anaerobic	Jousimies-Somer <i>et al.</i> 2002
KVLB	Laked blood, kanamycin, vancomycin	Gram-negative anaerobes	0, -4, -6	Anaerobic	Jousimies-Somer <i>et al.</i> 2002
BIF	Tomato juice, hemin, maltose	Bifidobacteria	0, -4, -6	Anaerobic	Sutter <i>et al.</i>
MRS	Dextrose, ammonium citrate	Lactobacilli	0, -5, -7	Anaerobic	Jousimies-Somer <i>et al.</i> 2002
CCFA	Fructose, egg yolk, cycloserine, cefoxitin	<i>Clostridium difficile</i>	0, -3	Anaerobic	Jousimies-Somer <i>et al.</i> 2002
NEYA	Egg yolk, neomycin	<i>Clostridium</i> spp.	0, -4	Anaerobic	Jousimies-Somer <i>et al.</i> 2002
Blood	Sheep blood	Total aerobic counts	0, -3, -5, -6, -8	5% CO <sub>2</sub>	MacFaddin 1985
CLED	Cystine, lactose, electrolyte deficient	<i>Enterobacteriaceae</i> , Streptococci	0, -4	Ambient air	MacFaddin 1985
BE	Bile, esculin	Enterococci	0, -4	Ambient air	MacFaddin 1985
Sabouraud	Chloramphenicol	Yeasts	0	Ambient air	MacFaddin 1985

jars filled by the evacuation-replacement technique (Anoxomat WS8000, Mart B.V., Lichtenvoorde, Netherlands) with gas mixture (90% N<sub>2</sub>, 5% CO<sub>2</sub>, 5% H<sub>2</sub>). The exposure time to air was minimized throughout the procedure with careful planning and preparations, including the use of prereduced dilution broth and agar plates kept in anaerobic conditions 18 h prior to their use.

The total counts and main groups of aerobic and anaerobic bacteria and yeasts were enumerated (the detection limit 10<sup>2</sup> CFU/g), different colony morphotypes were recorded with a stereomicroscope and isolated and identified by established methods. The isolates were picked according to differing morphologies, not on the amount of colonies on the plate or assumed importance of a finding. The identification scheme included aerotolerance testing, gram staining, spot and screening tests (catalase, indole, nitrate, lecithinase, lipase, pigment), special antimicrobial potency disk patterns (vancomycin, kanamycin, colistin, sodium polyanethol sulfonate, penicillin, oxgall), biochemical tests (carbohydrate fermentation, enzyme detection) and metabolic end-product analysis by gas-liquid chromatography after peptone-yeast extract glucose broth culture (Jousimies-Somer *et al.* 2002, Murray *et al.* 2003). The presence of *C. difficile* common antigen and toxin A in faeces was determined with a commercial kit (Triage *C. difficile* Panel, Biosite, San Diego, CA) according to the manufacturer's instructions. For unclassified clostridia resembling *C. difficile*, genes encoding 16S rRNA were amplified and sequenced with the primers fD1 Mod and 533r by the method of Jalava *et al.* (1995) and compared to the GenBank database. The microbial composition and counts between different sampling days, different treatment groups and between corresponding jejunal and faecal samples were compared.

## **5. Identification, susceptibility testing and PFGE typing of coliforms**

From each sample ten colonies or, if less, as many as possible of coliformic colonies (aerobic fermentative gram-negative rods) were isolated from Blood agar and CLED agar plates with optimal growth, and sub-cultured for further identification (Murray *et al.* 2003) and susceptibility testing (NCCLS 2002). The isolates were picked at random but all visibly different colonies were included to get a representative selection of the

coliform population. The resistant isolates may not present the most common morphologic phenotype nor differ by e.g. lactose fermentation profile (Levy *et al.* 1988). The species level identification of coliforms was performed with spot tests (catalase, indole, oxidase), an individual diagnostic tablet of  $\beta$ -glucuronidase (Rosco, Taastrup, Denmark) and with the Api 20E kit test (bioMérieux, Marcy l'Etoile, France).

For all isolated coliforms the resistance to several classes of antimicrobials including penicillins (ampicillin 10  $\mu$ g), carbapenems (meropenem 10  $\mu$ g), tetracyclines (tetracycline 30  $\mu$ g), folate pathway inhibitors (trimethoprim 5  $\mu$ g, trimethoprim/sulfamethoxazole 24  $\mu$ g), aminoglycosides (gentamicin 10  $\mu$ g), quinolones (ciprofloxacin 5  $\mu$ g), and cepheims (cephalothin 30  $\mu$ g, cefotaxime 30  $\mu$ g) was tested (Oxoid, Hampshire, England). The susceptibility was determined using the disk diffusion method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS 2002) on Müller Hinton agar inoculated with swabs dipped in bacterial suspension of 0.5 McFarland with 16-18 h incubation. *E. coli* ATCC 25922 was used as a control strain.

The selected *E. coli* isolates were typed using pulsed-field gel electrophoresis (PFGE) as described by Saari *et al.* (2001). The selected strains presented all different resistance patterns present in each dog at each time point. Bacterial suspension with  $A_{600}$  of 0.300 was done in TEN buffer and embedded in 1.8% agarose (InCert Agarose, BioWhittaker Molecular Applications, Inc., Denmark) to prevent mechanical damage to the bacterial chromosome, lysed with proteinase K (0.5 mg/ml) (Roche, Mannheim, Germany) in ES buffer overnight at +50°C, washed to remove the protein digestion products, and the DNA was digested at +37°C overnight with restriction endonuclease *Xba*I (New England Biolabs, Beverly, MA) that cuts DNA infrequently. The blocks were run in 1% agarose gel (Seakem Gold Agarose, BioWhittaker Molecular Applications, Inc., Denmark) in 0.5 x TBE buffer (6V/cm, 120°, 14°C, 21h, pulse times 5-40s) to resolve the restriction fragments into different patterns of discrete bands. The electrophoretic apparatus switches the direction of the current as programmed causing the fragments to reorientate between movements. As the swiftness of the reorientation is dependent on the size of the fragments this intensifies the separation of fragments with small difference in size. The

gels were stained with ethidium bromide (10µl EtBr / 200ml TBE, 30 min) and photographed under UV-light (Alpha Imager, Alpha Innotech, San Leandro, CA). The images were analysed using the Bionumerics version 3.0 cluster analysis with Dice similarity indices (1% position tolerance, UPGMA) (Applied Maths BVBA, Austin, Texas). The genetic relatedness of the strains based on PFGE profiles (fingerprint patterns) was interpreted as recommended by Tenover *et al.* (1995).

## **6. TGGE (temperature gradient gel electrophoresis)**

TGGE was used to assess the bacterial profile and diversity with a molecular approach, based on the sequence variability of the V6 to V8 regions of the 16S rRNA. TGGE was performed as described by Muyzer *et al.* (1993) using primers P16S968GCF and P16S1401R. Briefly, 50 mg of faeces was homogenised and the bacterial DNA was extracted. PCR reaction was performed using Invitrogen's *Taq* DNA polymerase kit and PTC-200 PCR system (MJ-Research). A temperature gradient was established in polyacrylamide gel in parallel to an electric field. The gels were run (200 V, 60°C) in 1.5% (wt/vol) agarose gel using TGGE MAXI system (Biometra, Germany). The amplified DNA fragments migrate differently according to their size and sequence specific melting behaviour (thermal stability) to the point where the conformation of the double stranded DNA changes. As the sequences in TGGE are all the same length the fragments separate in sequence specific manner, and a profile reflecting the prominent bacterial content of the sample is achieved. Fragments containing more A-T pairs are more vulnerable to temperature than those containing C-G pairs. To prevent the double stranded DNA to denature into single stranded DNA, a C-G rich segment is added to one of the primers. The gels were silver stained with AgNO<sub>3</sub> by the method of Cairns and Murray (1994). The individually unique TGGE profiles obtained from the same individual on different sampling days were compared using similarity percentages and constructing dendrograms using GelCompar II software (Applied Maths, Belgium).

## **7. PCR for detecting TEM gene**

The emergence of ampicillin resistance was evaluated also by performing quantitative real-time PCR of the TEM  $\beta$ -lactamase gene by quantifying the percentage of TEM

producing bacteria of the total number of bacteria as proportions of DNA in the faecal samples. The PCR was designed for V6-V8 region of the 16S rRNA gene using TemA, TemB, TemE and TemH primers and SYBR green (Molecular Probes, AA Leiden, Netherlands) as fluorogenic marker. The bacterial total count was set as 100% and the amount of Tem sequences was compared to the total bacterial DNA. SYBR Green is a highly sensitive (for low numbers of target DNA amplification product) fluorescent dye specific for double-stranded DNA. During quantitative PCR reaction, SYBR Green dye binds to the amplified double-stranded PCR products and emits a fluorescent signal. Detection of this signal enables direct quantitation of amplified DNA by comparing the fluorescence to known standards.

## **8. Measurement of ampicillin concentration**

In addition to microbial analyses, the serum (quantification limit 1.0 µg /ml) and jejunal (0.5 µg /ml) ampicillin levels were monitored in samples collected 1 h after the first antibiotic administration of the day. The ampicillin analysis was performed using high performance liquid chromatography (HPLC) by modification of the method of Vree *et al.* (1978). HPLC analysis is based on the different migration rates of different compounds in particular chromatographic conditions. A characteristic peak at expected retention time is observed in a chromatograph for each compound in the injected sample. The quantification of the compound the peak presents is calculated from the area under the peak using standard calibration curve with known amounts of the compound.

The jejunal samples were frozen (-70°C) until analysis when they were thawed, mixed by vortex and centrifuged (4°C, 1800 g, 15 min). The tubes were placed on ice and the supernatant was transferred to a cooled glass beaker, diluted in cooled NaCl, filtered through 0.22 µm filters and frozen (-70°C) until analysed by HPLC. The serum samples were centrifuged (1000g, 15 min) and frozen until analysed. A Hewlett Packard 1100 HPLC instrument with a diode-array detector was used as follows: Supelco Discovery RP Amide C16 column, 2 mobile phases at a flow-rate of 1.0 ml/min (96 % phosphate buffer, pH 6.0 and 4 % acetonitrile for quantitative determinations, 96 % ammoniumacetate, pH 5.2 and 4 % acetonitrile for spectral identification), detection at 229 nm for ampicillin and 296 nm for internal standard (benzylpenicillin procain).

## **9. Statistical analysis**

The statistical analyses for culture-based methods were performed using the SPSS 11.5 (SPSS, Chicago, IL, USA) and Epi Info 6 Statcalc (CDC, Atlanta, GA USA) software for Windows. The pH values of the samples and number of isolates in the 2 sample types were compared by the paired samples t-test and frequencies of different bacteria by Statcalc single table tests. The differences between the bacterial counts were assessed by the Wilcoxon signed rank test (non parametric). A *P*-value <0.05 was considered statistically significant.

For the molecular methods the data analyses were performed with Statistical Analysis System (SAS) software (version 8.1, 2000; SAS Institute Inc.). The PROC MIXED program in SAS software was used to analyse the repeated measurements of the percent similarities of the faecal flora determined by TGGE. The nonparametric analysis of variance and ANOVA were used to compare the ampicillin concentrations and TEM gene levels. A *P*-value < 0.01 was considered statistically significant.

## RESULTS

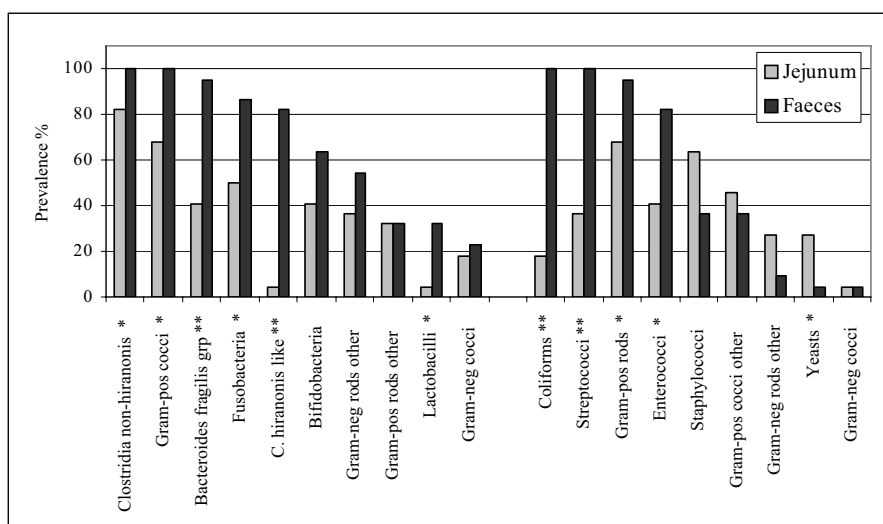
### 1. Jejunal versus faecal cultivable microbiota (I)

The microbial findings of jejunal chyme and corresponding faecal samples were compared. Jejunal fluid samples varied markedly in viscosity, texture, and color (mucous/running, reddish/brown/greenish). The odor of the fluid was mild and often slightly sweet. All samples were culture-positive: all growth in jejunal samples was recorded and identified, while from faecal samples only bacterial groups in dilutions  $>10^{5-6}$  could usually be isolated and identified. Because of relatively low bacterial counts, different colony types were technically easier to isolate from the jejunal than faecal samples. Significant differences between the two sample types were found both qualitatively and quantitatively. The total cultivable bacterial counts in the jejunum were outnumbered by the counts in faeces ( $10^{2-6}$  vs.  $10^{8-11}$  cfu/g, respectively). Notably, bacterial counts in 9% (2/22) jejunal baseline samples reached  $10^6$  cfu/g and 27% (6/22) reached  $10^5$  cfu/g with varied dominating species. Anaerobes covered a significantly lower proportion of the total bacterial count in the jejunum compared to faeces (48% vs. 79%, respectively,  $P=0.004$ ), and significantly less bacterial genera/species were found in the jejunum than in faeces (7 vs. 12, respectively,  $P<0.001$ ).

The proportions of different cultivable bacteria differed significantly between the jejunal and faecal samples as presented in Figure 1 and 2. Altogether 25% of the bacteria found in the jejunum were not detected in the corresponding faecal sample, or 45% of the faecal findings in the jejunum. In addition, several microbes such as staphylococci, other catalase-producing gram-positive cocci, non-fermentative gram-negative rods and yeasts were more frequent in the jejunum than in faeces, and some bacteria (neisseria, micrococci), most likely of oropharyngeal origin, were detected only in the jejunum. Although gram-negative organisms had on average a higher prevalence in the faecal than jejunal samples (53% vs. 28%), the proportion of gram-negative organisms of the total count was higher in the jejunum than in faeces (28% vs 3%). The dominating cultivable species (those with highest counts) were the same in the corresponding faecal and jejunal sample in 5/22 (23%) dogs. In faeces, the dominating species were similar between

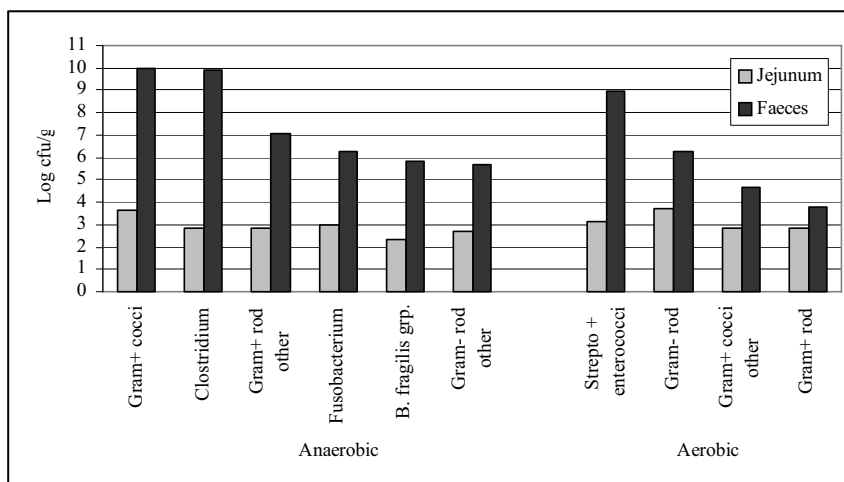


different dogs (anaerobic gram-positive cocci in 10 and streptococci in 5 dogs), whereas the dominating species isolated from jejunal chyme varied between the dogs (anaerobic gram-positive cocci in 4, staphylococci in 4, aerobic gram-positive rods in 4 dogs). A group of unclassified clostridia (“*Clostridium hiranonis*- like” organisms) was found in just one jejunal sample but in 18/22 (82%) of faecal samples ( $P<0.001$ ). The isolates resembled *C. difficile* biochemically whereas the 16S rRNA sequence analysis was 99% identical to *C. hiranonis* but the isolates were biochemically different from *C. hiranonis*. Among the *Bacteroides fragilis* group *B. fragilis* was the dominating species in the jejunum while in faeces it was just one species among others, *B. vulgatus* and *B. caccae* being at least as common. Out of the 166 faecal coliform isolates *E. coli* covered 98% while of the 9 coliform isolates from the jejunum 6 (67%) were *Citrobacter* spp. and 2 (22%) *E. coli*.



**Figure 1.** Prevalence of cultivable anaerobic and aerobic/facultative bacteria and yeasts in the jejunum and faeces at baseline (Study I). Epi Info 6 single table test:

\*  $P<0.05$ , \*\*  $P<0.001$ .



**Figure 2.** Median counts (log cfu/g) of different cultivable bacterial groups in the jejunum and faeces at baseline (Study I).

## 2. Stability of the cultivable intestinal microbiota (I)

Five consecutive samples from 7 of the dogs, collected during a month, were compared with each other. The predominant jejunal species varied between the dogs and between the consecutive samples while the faecal microbiota was more stable and more similar between the dogs. The findings in the consecutive samples were found repeatedly in 42% of the jejunal samples and in 60% of the faecal samples. In faecal samples the variation in time was biggest among subdominant species close to the detection limit while in jejunal samples all species varied. Several microbial groups or species such as streptococci, yeasts, gram-negative anaerobic and aerobic cocci, gram-positive anaerobic cocci, *C. perfringens* and staphylococci were never found in all consecutive jejunal samples of a dog. In faecal samples dominant species remained stable though their counts fluctuated.

## 3. Effects of ampicillin and prevention of them by TRBL (II, III)

The effects of ampicillin were followed in the group receiving ampicillin + placebo (Amp/Pla) (Table 1). Significant changes in the cultivable microbial composition and in the resistance rates were recorded in this treatment group, while oral  $\beta$ -lactamase preserved the faecal microbiota during the ampicillin treatment, monitored as significant

differences between the groups receiving ampicillin + placebo and ampicillin + TRBL (Amp/TRBL), and, on the other hand, no marked differences between Amp/TRBL and placebo + placebo (Placebo) groups. The effects with similar differences between treatment groups were found by all methods used: culture, susceptibility testing, TGGE and TEM-PCR.

**Table 1.** The effects of ampicillin in the Amp / Pla group.

<b>Method</b>	<b>Effect detected in Amp/pla group</b>
Bacterial culture	Altered bacterial profiles, decreased total counts
Susceptibility testing	Significant increase in ampicillin (multi)resistance
TGGE	Altered bacterial profiles (drop in similarity percentage)
TEM-PCR	Increased amount of TEM resistance gene
High performance liquid chromatography (HPLC)	High serum and jejunal ampicillin concentrations

### **3.1. Changes detected by culture (II, III)**

During the intervention the total faecal count of cultivable aerobes decreased significantly in the Amp/Pla group ( $P=0.003$ ) whereas in the Amp/TRBL group the total counts remained fairly stable. Although the decrease in the anaerobic total count in the Amp/Pla group was not significant compared to the baseline, the anaerobic total count was significantly lower than in the Amp/TRBL group during the treatment ( $P=0.041$ ). Decreased counts were recorded for streptococci, enterococci, clostridia and anaerobic gram-positive cocci, while aerobic and anaerobic gram-negative rods increased their proportion of the total count.

### **3.2. Microbial shifts detected by TGGE (II)**

During the treatment the overall percentage similarity of the faecal microbiota, determined by TGGE, dropped to a significantly lower level in the Amp/Pla group (38-53%) compared to the Amp/TRBL and Placebo groups (78-85%) ( $P<0.001$ ).

### **3.3. Ampicillin concentrations (II)**

$\beta$ -Lactamase had no effect on the ampicillin concentrations in serum; the concentrations were similar in the groups receiving ampicillin and placebo or ampicillin and TRBL (mean 17.7 vs. 18.0  $\mu\text{g/ml}$ , respectively). No ampicillin was detected in the Placebo group.

The ampicillin concentrations in the jejunum were significantly higher in the group receiving ampicillin and placebo than in the group receiving ampicillin and TRBL (mean 26.3 vs. 1.5  $\mu\text{g/ml}$ , respectively,  $P < 0.0001$ ). In the latter group  $\beta$ -lactamase had degraded the intestinal antibiotic residue.

### **3.4. Change in TEM gene levels (II)**

The TEM gene levels (determined as the mean percentage of TEM gene of the total amount of DNA) were very low or undetectable in all dogs at baseline. During the treatment the proportion of TEM gene reached a significantly higher level in the Amp/pla group ( $>0.15\%$ ) than in the other groups ( $<0.025\%$ ) ( $P < 0.001$ ).

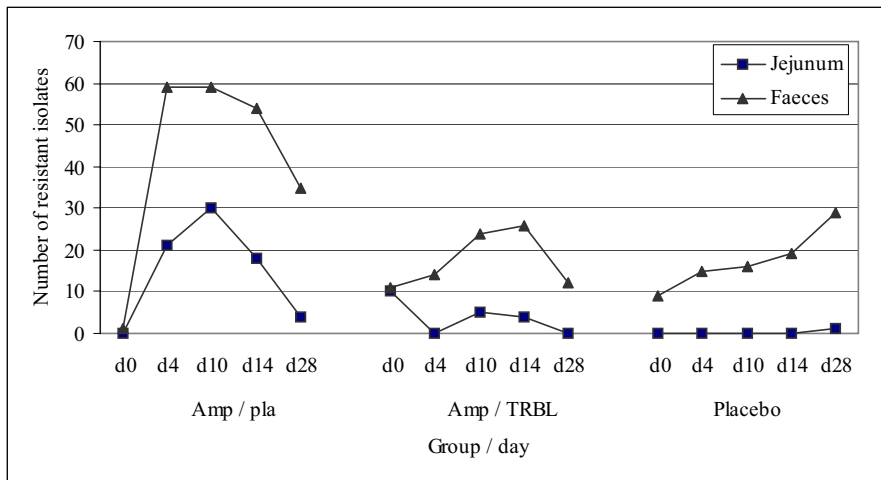
## **4. Antimicrobial resistance in coliforms (II, III, IV)**

Altogether 982 (860 faecal, 122 jejunal) coliform isolates were collected, identified and tested against 9 antimicrobials. *E. coli* covered 87% (106/122) of the jejunal and 99% (855/860) of the faecal isolates. A total of 22 different resistance patterns, with 12 patterns displaying ampicillin resistance, were identified. Each sample displayed 2-3 (ranging between 1-6) different patterns. The 5 most common patterns covered 94% of the faecal and 77% of the jejunal isolates.

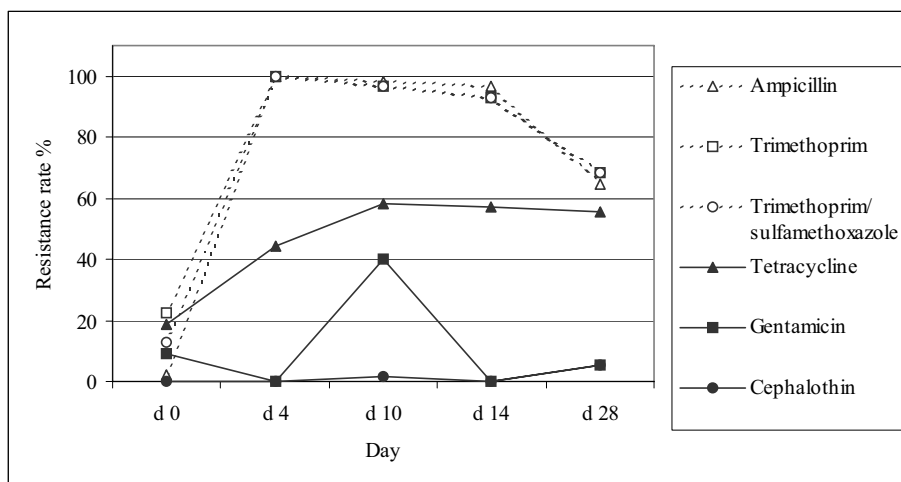
### **4.1. Emergence of resistance in coliforms (II, III, IV)**

An increase in ampicillin resistance (multi-resistance) was detected in all treatment groups but the resistance rate was significantly higher in the Amp/Pla group than in the

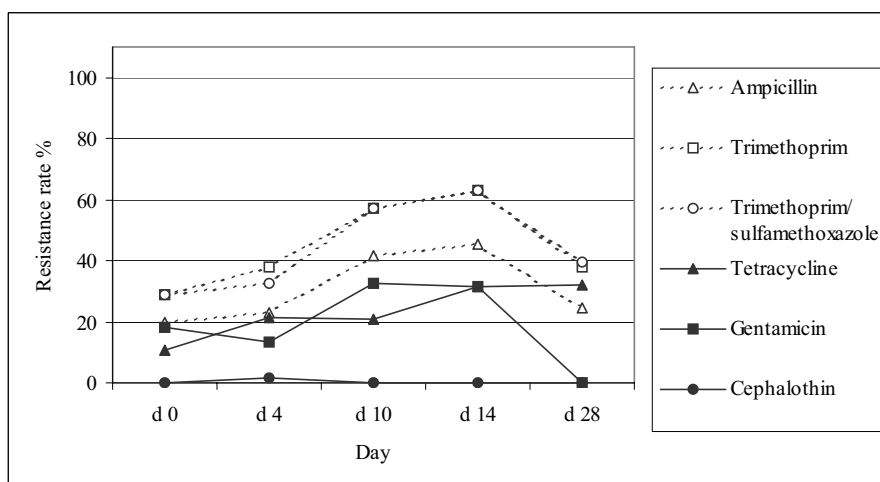
other groups ( $P<0.001$ ) (Figure 3). Ampicillin-resistant isolates were frequently resistant also to trimethoprim, trimethoprim/sulfamethoxazole, tetracycline and occasionally to cephalotin. None of the isolates were resistant to ciprofloxacin, meropenem, or cefotaxime. The overall resistance rates in the 3 treatment groups are presented in Figures 4a-c. In the jejunal samples the pattern of increasing resistance was similar to faeces but, additionally, a significant increase was detected also in the number of jejunal coliforms available. During the treatment a total of 68 jejunal coliform isolates were obtained in the Amp/Pla group while in Amp/TRBL and Placebo groups there was no increase in the number of coliforms and only 16 and 15 isolates were obtained during the treatment, respectively.



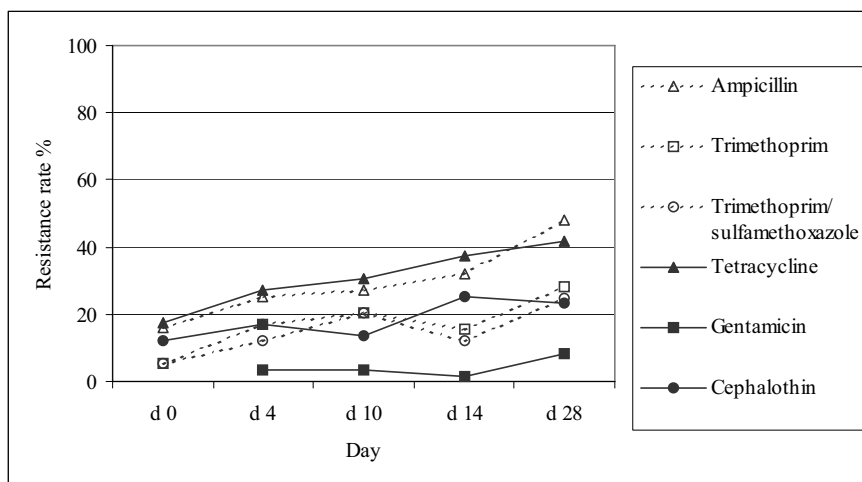
**Figure 3.** Number of ampicillin-resistant *E. coli* isolates from the jejunum and faeces in the treatment groups during the intervention.



**Figure 4a.** Resistance rates of coliforms during the intervention in the 3 treatment groups: Amp / Pla group.



**Figure 4b.** Amp / TRBL group.



**Figure 4c.** Placebo group.

#### 4.2. Assessing the relatedness of coliforms (IV)

The relatedness of coliform isolates was determined using the identification results and resistance patterns in combination with PFGE typing. Selected 402 (339 faecal, 63 jejunal) *E. coli* isolates were typed by PFGE and 25 different PFGE types were identified. Each dog harboured on average 5 (ranging between 2-8) different PFGE types during the 28 days' study period. The dogs shared several PFGE types. Ampicillin resistance was found in 6 different PFGE types in 9 different combinations of co-resistance (Table 2). None of the PFGE types was widely spread between the dogs in the different treatment groups but certain ampicillin-resistant PFGE types (F, G, E, A) were enriched in the Amp/Pla group where at least one new ampicillin-resistant PFGE type appeared in all dogs. In the Amp/TRBL group half of the dogs carried ampicillin resistant strains at baseline and during the treatment but among the other half no resistant strains were detected. In Placebo group new resistant strains appeared in 3 dogs during the treatment. Apart from the two strains in a single dog that seemed to acquire resistance during the Amp/pla treatment, the resistant strains enriched were most often selected from pre-existing resistant strains.

Resistance pattern	PFGE type	Isolated from group		
		Amp / Pla	Amp / TRBL	Placebo
Fully susceptible	A, B, C, D, H, I, J, K, M, P, Q, R, S, T, U, V, X, Z, AA, ff*	+	+	+
Amp, Tet, Tmp, Sxt	G, E, E <sub>1</sub>	+	+	+
Amp, Gen, Tmp, Sxt	A, D, F, F <sub>1</sub> , F <sub>2</sub>	+	+	+
Amp, Cef, Tet, Tmp, Sxt	G	+	-	-
Amp, Cef, Tet, Tmp	ff*	-	-	+
Amp, Cef, Tet	ff*	-	-	+
Amp, Gen, Tet, Tmp, Sxt	A	+	-	-
Amp, Cep, Tmp	Q	-	+	-
Amp, Tet	G, ff*	-	+	+
Amp, Tmp, Sxt	G	-	+	-
Tet, Tmp, Sxt	A, E, L, P	+	+	+
Tet	A, BB, Y	+	+	-
Gen, Tmp	F	+	-	-
Cef, Tet	BB	+	-	-
Tmp, Sxt	L	-	+	-
Tmp	A	-	+	+
Sxt	I	-	+	-
Gen	D, O	-	-	+
Cef	S	-	-	+

**Table 2.** Distribution of resistance patterns and PFGE types of the *E. coli* isolates. \*ff, untypeable. Amp, ampicillin; Tet, tetracycline; Tmp, trimethoprim; Sxt, trimethoprim/sulfamethoxazole; Gen, gentamicin; Cef, cephalothin.



## DISCUSSION

### Characterisation of jejunal and faecal microbiota by culture

The isolation and identification of all species present in a faecal sample is thought to be extremely demanding if not impossible. Regardless of the methodology chosen, only the predominant populations of the cultivable microbial groups can most often be monitored. Although the selectivity of currently available agar media is limited and requires further characterisation and identification methodology, our culture method proved to be sensitive enough to detect differences between the two sample types regardless of the restricted use of the species level identification.

In the present study the nature of the small intestinal microbiota, differing markedly from that of the colon, was characterised as fairly simple and variable, consisting of only few species at a time with fluctuating counts compared to the rich and stable faecal microbiota. All samples were frozen (-70°C) without delay and processed later in parallel, however, the effect of freezing on the microbes of jejunal samples with varying texture has not been fully assessed. The jejunal bacterial counts in some of these healthy beagles crossed the typical limit for small intestinal overgrowth ( $10^5$  cfu/ml) (Corazza *et al.* 1990, Stotzer *et al.* 1998). The counts were not so high repeatedly but all jejunal samples grew bacteria confirming the unreliability of diagnosis of overgrowth based on a single culture. In addition, this finding questions the feasibility of such a definitive limit. On the other hand, the jejunal samples in the present study were obtained without anesthesia shortly after feeding and may have been therefore exceptionally productive yielding high bacterial counts including anaerobes. It cannot, however, be excluded that the permanent fistula may in some individuals have had an effect on the intestinal motility and bacterial counts.

The quantitative and qualitative relations within the cultivable microbiota were significantly different between the two sample types. Our observation is in agreement with previous findings reporting higher ratios of aerobes and differences in frequencies of gram-negative organisms in the upper human gut (jejunum, ileum and caecum) compared

to the colon and faeces (Gorbach *et al.* 1967, Justesen *et al.* 1984, Marteau *et al.* 2001). Oropharyngeal microbes have been reported to dominate in jejunal biopsies of humans (Sullivan *et al.* 2003), whereas *E. coli* and streptococci were the most common findings in the jejunum of 35 mongrel dogs (Thompson *et al.* 1998). Higher or comparable counts of fungi, staphylococci and lactobacilli have been reported in the human ileum than faeces (Gorbach *et al.* 1967). Similarly we found staphylococci, yeasts and also other catalase-producing cocci and non-fermentative gram-negative facultative rods more frequently in the canine jejunum than in faeces but less lactobacilli. Additionally, some species isolated from jejunal samples were undetected in faeces. Marteau *et al.* (2001) reported facultative bacteria to be as numerous as anaerobes in the human caecum. This seems to be true in the jejunum as well, as shown in the present study. *B. fragilis* proved to be the most frequent species of the *B. fragilis* group in the canine small intestine but not in faeces. In agreement with our observation, *B. fragilis* has been reported to be the most frequent isolate from mucosal surfaces of humans but not in colon contents (Namavar *et al.* 1989). In the present study the canine jejunal microbiota was also found to be less stable than the faecal microbiota. The canine duodenal microbiota monitored by DGGE profiles has been shown to differ more between individual dogs than between samples from the same dog (Suchodolski *et al.* 2004). The canine fecal bacterial DGGE profiles have individually unique and stable characteristic banding patterns (Simpson *et al.* 2002).

It is noteworthy that we found no *C. difficile* in contrast to some other animal studies (Marks *et al.* 2002, Buogo *et al.* 1995) but we detected instead a group of unclassified clostridia resembling *C. difficile* as part of the dominating faecal microbiota. In addition to strict anaerobes, facultative streptococci were among the dominating microbiota. Also, we found bifidobacteria more often than lactobacilli (in 64% vs. 32% of the dogs, respectively) while two recent studies on labrador and beagle dogs reported no bifidobacteria but numerous lactobacilli (Buddington 2003, Greetham *et al.* 2002). The lactic acid bacteria in fistulated dogs were recently characterised by Rinkinen *et al.* (2004) with *Streptococcus alactolyticus* dominating, followed by *Lactobacillus murinus* and *L. reuteri*.

### **Comparison of culture to molecular methods**

Though molecular methods have brought new aspects to the phylogenetic and quantitative relations of different bacteria in the gut, culture-based and molecular-based profiling may not be contradictory and give often in general somewhat similar overview. Culture-based analyses may, however, give higher qualitative and quantitative fluctuations especially in the faecal microbiota compared to molecular methodology. Similar to our study, the vast fluctuation in canine small intestinal microbiota compared to faeces has been reported by both culture and DGGE reflecting genuine variation rather than weakness in sampling or analysis methodology (Willard *et al.* 1994, Delles *et al.* 1994, Harmoinen *et al.* 2001, Suchodolski *et al.* 2004). We found by culture in the canine faeces a relatively low mean proportion of *Bacteroides* (0.3%), a high proportion of anaerobic gram-positive cocci (30%) and clostridia (37%), a relatively high proportion of streptococci (19%) and relatively low proportion of bifidobacteria (4%) and enterobacteria (1%). Culture negativity or limited number of microbial findings may not always give an accurate picture of the number or consortia of microbes present. For example, cultures and microscopic examinations of oesophageal washes and biopsies have provided poor microbial yields but microbes in oesophageal biopsies detected by sequence based broad-range 16S rDNA are by a large majority (82%) cultivable bacterial species (Pajecki *et al.* 2002, Pei *et al.* 2004).

### **Ampicillin-induced changes**

In the present study ampicillin was found to induce significant qualitative and quantitative changes and emergence of multi-resistant coliform strains among the canine intestinal microbiota. These phenomena have been well documented for ampicillin and several other antimicrobials also in previous human studies (Nord 1986a, Black *et al.* 1991, Garau *et al.* 1999, Shanahan *et al.* 1994, Huovinen *et al.* 1995, Dradenovich *et al.* 2004). Among human faecal enterobacteria *E. coli* is the main carrier of resistance (Österblad *et al.* 2000). In the present study ampicillin resistance was most often combined to trimethoprim and trimethoprim/sulfamethoxazole resistance in canine strains. A similar combination of resistance occurs frequently, since transmissible

plasmids coding resistance to ampicillin and trimethoprim are common in human *E. coli*, and selective pressure for either is likely to select resistance to both drugs (Amyes 1989, Magee *et al.* 1999). As shown previously, the screening of coliform susceptibility is a feasible and sensitive method to follow the steps in the emergence of resistance (Corpet 1993). In the present study, to intensify the resistance screening, 10 isolates representing all colony morphotypes per sample were tested for resistance. Further, to overcome biases due to the culture method, molecular methods were used to follow the changes in resistance rates monitored as amounts of TEM gene. In addition, several samples during the treatment were analysed.

### **Resistance within coliform populations**

PFGE typing revealed a considerable genetic heterogeneity within the ampicillin-resistant canine *E. coli* populations. Though digestion of DNA with more than one restriction enzyme could have increased the discriminatory power of the PFGE method and separated strains considered identical, the present analysis with one enzyme (*Xba*I) was successful in discriminating numerous genetically different clones with varying frequencies within a large number of related isolates in the three treatment groups. Dogs shared several clones but no single resistant strain was widely spread. Since the resistant strains were most often selected from the pre-existing microbiota or spread between dogs, and were genetically heterogeneous, several resistant types co-existed. The selection of resistant strains from the resident microbiota is thought to be more common than acquisition of a new resistance determinant in strains previously susceptible (Guyot *et al.* 1999, McDonald *et al.* 2001) as was the case also in the present study.

Heterogeneity, constant shifts between strains within *E. coli* populations and sharing of common types has been reported in humans, dogs and cattle (Levy *et al.* 1988, Avery *et al.* 2004, Akiba *et al.* 1999, Münnich & Lübke-Becker 2004, Dradenovich *et al.* 2004). The appeared resistant strains replaced the PFGE types present at baseline in the dogs receiving ampicillin without  $\beta$ -lactamase but not in the other two groups.

### **Prevention of ampicillin-induced changes by TRBL**

Orally administered  $\beta$ -lactamase did not affect the ampicillin level in serum but degraded ampicillin in the intestine and prevented the ampicillin-induced changes in the intestinal microbiota. Colonisation resistance was reduced and multi-resistant coliforms colonised the jejunum only in dogs treated with ampicillin without  $\beta$ -lactamase but not in dogs treated with ampicillin with  $\beta$ -lactamase. The slight increase in resistance rates detected in the Amp/TRBL and Placebo groups could be due to the cross-contamination and spread of resistance determinants from the dogs in the Amp/pla group, since the dogs were not totally isolated from each other. Other possible sources of resistance determinants include food, environment, kennel facilities and personnel as reported also in other studies (Corpet 1987, Avery *et al.* 2004, de Graef 2004). Concurrent administration of antibiotic and  $\beta$ -lactamase could provide a new strategy to limit the emergence of resistance and spread of nosocomial pathogens, and reduce antibiotic-associated diarrhoea. In addition to ampicillin,  $\beta$ -lactamase has shown to be applicable to inhibit piperacillin-induced changes (Stiefel *et al.* 2003).

Commensal microbes gradually overgrow the resistant strains when the selection pressure is absent (Trott *et al.* 2004). The restoration of the microbiota may, however, be slower than previously thought. The antibiotic-associated alterations in the microbiota can be detected for months or even up to 3 years after a single antibiotic course (Maxwell *et al.* 2002, Mangin *et al.* 1994, Sjölund *et al.* 2003). Antimicrobial resistance determinants are known to be transferable among the commensal microbiota (Gulay *et al.* 2000, Yates *et al.* 2004). In the present study, the microbiota had not recovered to the baseline configuration 2 weeks after the treatment. Although the bacterial counts and composition, TGGE profiles and TEM gene proportions resumed levels close to the baseline, the enriched or appeared ampicillin-resistant PFGE types in the Amp/pla and Placebo groups were still present and the proportion of resistant isolates was higher than at baseline.

### **Future aspects**

The next phase in studying combined ampicillin and  $\beta$ -lactamase administration would be a study trial in humans. Further phases in developing means to prevent antimicrobial

resistance using  $\beta$ -lactamase could include development of new  $\beta$ -lactamases targeted to other  $\beta$ -lactams.

## KEY FINDINGS AND CONCLUSIONS

To protect the indigenous microbiota in a safe and convenient manner the composition and functions of the microbiota should be well known and understood, and the desired changes, or their absence, as well as their magnitude and dimensions should be closely monitored with suitable methodology. The present study was carried out in order to develop the understanding of the jejunal microbial composition and stability, and to specify the dissimilarities between the jejunal and faecal microbiota. Another main focus of the study was to evaluate the ability of enzymatic inactivation of intestinal antimicrobial residues to inhibit antibiotic-induced adverse effects on the microbiota.

The key findings were:

I        Significant microbial differences between the jejunal and faecal findings were recorded in regard to the bacterial counts, species composition, species variability, stability, relations of gram-negative to gram-positive organisms and relations of aerobic to anaerobic microbes.

II       Ampicillin-induced changes among the intestinal microbiota were inhibited to a significant extent by simultaneous oral  $\beta$ -lactamase administration.

III      Ampicillin treatment without simultaneous  $\beta$ -lactamase administration induced the emergence of multi-resistant strains displaying numerous combinations of co-resistance and significantly increased the colonisation of the jejunum with coliforms. The increase in resistance was markedly inhibited and the jejunal colonisation resistance was preserved when ampicillin treatment was combined to  $\beta$ -lactamase.

IV      The emergence of resistance among intestinal coliforms was due to selection and/or dissemination of several genetically different strains rather than acquisition of resistance in previously susceptible strains or spread of single or few resistant strains between the dogs in different treatment groups.

## **Conclusions**

The small intestinal microbiota has individually unique features in regard to counts, species composition and variability of the microbes, differing markedly from those of faeces. Orally administered  $\beta$ -lactamase was shown to have potential in clinical use by inhibiting the emergence of resistance and preserving the microbiota during antimicrobial therapy.  $\beta$ -Lactamase can be beneficial in inhibiting the emergence and enrichment of resistant strains and in maintaining the colonisation resistance in jejunum during ampicillin-induced selection pressure.



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A handwritten signature in dark ink, appearing to read 'S. J. Ahti'.

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